

Two-dimensional Gel Electrophoresis of Rat Liver Nuclear Washes, Nuclear Matrix, and hnRNA Proteins

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ABSTRACT The proteins of rat liver cytoplasm, nuclear washes, matrix, membrane, heterogeneous nuclear (hn)RNA proteins and chromatin were examined by two-dimensional gel electrophoresis. The inclusion in the gels of six common protein standards of carefully selected molecular weight and isoelectric point allowed us to clearly follow the distribution of specific proteins during nuclear extraction. In the nuclear washes and chromatin, we observed five classes of proteins: (a) Exclusively cytoplasmic proteins, present in the first saline-EDTA wash but rapidly disappearing from subsequent washes; (b) ubiquitous proteins of 75,000, 68,000, 57,000, and 43,000 mol wt, the latter being actin, found in the cytoplasm, all nuclear washes and the final chromatin pellet; (c) proteins of 94,000, 25,000, 22,500, and 20,500 mol wt specific to the nuclear washes; (d) proteins present in the nuclear washes and final chromatin, represented by species at 62,000, 55,000, 54,000, and 48,000 mol wt, primarily derived from the nuclear matrix; and (e) two proteins of 68,000 mol wt present only in the final chromatin. The major 65,000–75,000-mol wt proteins seen by one-dimensional gel electrophoresis of nuclear matrix were very heterogeneous and contained a major acidic, an intermediate, and a basic group. A single 68,000-mol wt polypeptide constituted the majority of the membrane-lamina fraction, consistent with immunological studies indicating that a distinct subset of matrix proteins occurs, associated with heterochromatin, at the periphery of the nucleus. Actin was the second major nuclear membrane-lamina protein. Two polypeptides at 36,000 and 34,000 mol wt constituted 60% of the hnRNP. Approximately 80% of the mass of the nonhistone chromosomal proteins (NHP) from unwashed nuclei is contributed by nuclear matrix and hnRNPs, and essentially the same patterns were seen with chromatin NHP. The concept of NHP being a distinct set of DNA-bound proteins is unnecessarily limiting. Many are derived from the nuclear matrix or hnRNP particles and vary in the degree to which they share different intracellular compartments.

The study of the nonhistone chromosomal proteins (NHP) has been hindered by the tremendous diversity of these proteins with respect to size, charge, solubility, and function (for reviews, see references 23 and 40). The term "nonhistone" itself emphasizes that these proteins have historically been viewed more in terms of what they are not than what they are. They have been operationally defined as a heterogeneous group of proteins which along with histones, DNA, and various species of RNA, make up the interphase chromosome, or chromatin (18, 23). For many purposes this definition has been satisfactory. However, many studies on the solubility of nuclear acidic proteins in low ionic-strength buffers have demonstrated that nuclear proteins soluble in, for example, 0.35 M NaCl closely resemble the residual chromosomal nonhistones (30, 31, 35, 44,

45, 62, 87). This suggested that what many researchers have normally regarded as cytoplasmic or nucleoplasmic "contamination" of the chromosomal nonhistone fraction, are, in fact, loosely bound nonhistones. Furthermore, the studies of Comings and Tack (35), which demonstrated that most of the NHP (but not histones) could be removed from chromatin by repeated washing in buffers closely approximating physiological ionic strength, implied that perhaps less emphasis should be placed on regarding the NHP as a distinct class of proteins exclusively associated with chromatin, but rather that they should be considered in the broader context of being in dynamic flux between chromatin and the surrounding environment. Subsequently, other investigators have also addressed this point (16, 84, 86).

Additional support for this kind of thinking lies in fusion-type studies of eucaryotic cells which demonstrate the dynamic equilibrium between nucleus and cytoplasm of proteins that regulate cellular activity (19). Allfrey et al. (4) have shown that gene activation in lymphocytes treated with the plant lectin, concanavalin A, is accompanied by massive, yet selective, migration of protein from the cytoplasm to nuclear chromatin, and that not all of this protein is newly synthesized. Furthermore, steroid hormones have been shown to bind to target cell DNA via a cytoplasmic protein-hormone receptor complex (82).

One might infer from the studies on the solubility of NHP in low ionic-strength buffers that many of the proteins normally discarded during the preparation of chromatin may be important in chromatin structure and/or function. This would be a particularly important consideration when one is attempting certain kinds of quantitative assessments of the chromatin protein compliment (see, for example, reference 46). Therefore, to clarify the matter of the distribution of NHP during the preparation of chromatin, we examined, by two-dimensional gel electrophoresis (78), proteins of rat liver cytoplasm, whole nuclei, saline-EDTA and Tris washes of nuclei, and chromatin.

To further classify the subtypes of NHP we also examined the proteins of the nuclear matrix, nuclear membrane-lamina complex, and heterogeneous nuclear (hn)RNA proteins. The nuclear matrix forms the structural framework of the nucleus. It is composed of three parts: nuclear pore-lamina complex, nucleolar matrix, and intra-nuclear matrix (1, 9, 11, 13, 26, 32). The nuclear DNA is attached to all three parts. By whole-mount electron microscopy, part of the matrix is seen to be composed of fibrillar elements (32, 90) which we have termed matrixin. It has been suggested that hnRNA and its associated proteins are also intimately attached to the matrix (55, 71, 76). Thus, the nuclear proteins can be thought of as comprising two major classes, those associated with DNA (histones, gene regulatory proteins, polymerases, etc.) and those not primarily associated with DNA (nuclear membrane, nuclear matrix, nucleolus, and hnRNA proteins). Previous studies from this laboratory (26, 32), as well as work from Pederson's group (16, 84), using one-dimensional gel electrophoresis, indicate that the majority of the NHP fall into the second class.

In addition to forming a structural framework of the nucleus, some matrix proteins are also involved in formation of the synaptonemal complex and XY body (32) and some may play a role in metaphase chromosome structure (2, 3, 26, 27, 37, 66, 81) and formation of chromomeres. Those proteins involved in the nuclear membrane-lamina complex have been shown by immunocytological techniques to occur only at the periphery of the nucleus and to be phosphorylated and disassembled during late G2 phase (41, 48, 49, 63).

Because of these multiple functions, it is important to identify and classify the individual matrix proteins and determine which ones are primarily associated with the nuclear membrane-lamina complex, nucleolar matrix, intranuclear matrix, or hnRNP.

To support this analysis and comparison, we developed a system using internal protein markers of different molecular weight and isoelectric point which divide each two-dimensional gel into a 36-sector grid. Such a system has allowed us to accurately follow the distribution of specific proteins during nuclear extractions and to speculate on the distribution of such proteins within the cell.

MATERIALS AND METHODS

Preparation of Cytoplasm, Nuclei, and Nuclear Washes

Unless otherwise indicated, all operations were performed at 4°C. Four to five rats were killed by cervical dislocation and the livers perfused with ice-cold physiological saline. After removal, the livers were weighed, minced in 0.15 ml/g tissue of 10 mM Tris, pH 7.5, 3 mM CaCl₂ and 0.1 mM CdSO₄ containing freshly prepared soybean trypsin inhibitor (1 µg/ml), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM β-mercaptoethanol (βME). The tissue was homogenized with one stroke of a motor-driven Teflon pestle in a Bellco glass homogenizer (Bellco Glass, Inc., Vineland, N.J.), then a 1.2-vol of 2.4 M sucrose in 3 mM CaCl₂ and 0.1 mM CdSO₄ was added and mixed and the tissue was homogenized with nine additional strokes. After filtering through Nitex cloth (Tetko Inc., Elmsford, N.Y.; 3-7-210), ~20 ml of the homogenate was layered over 2.4 M sucrose and a crude gradient was formed with a Pasteur pipet in each of six SW27 tubes. The gradients were centrifuged at 16,000 rpm (35,000 g) in an SW27 rotor for 60 min. The nuclear pellets were suspended in 20 ml of SE buffer (0.075 M NaCl, 0.025 M EDTA in 10 mM Tris, pH 7.5 containing 5 mM βME and 0.1 mM PMSF). After incubating on ice for 10 min, the nuclei were centrifuged for 10 min at 2,000 rpm (650 g) in the Sorvall HB-4 rotor (DuPont Co., Instrument Products Div., Wilmington, Del.). This wash was repeated once, then followed by two identical washes in 10 mM Tris, pH 7.5, 5 mM βME, and 0.1 mM PMSF. Each wash supernate was further centrifuged for 1 h at 100,000 g in a 50 Ti-rotor before sodium dodecyl sulfate (SDS) treatment (see below). For cytoplasmic proteins, filtered homogenate from whole liver was first centrifuged for 30 min at 48,000 g, then the supernate was centrifuged for an additional 2 h at 100,000 g. The final supernate was then prepared for lyophilization in the same manner as with the SE and Tris washes.

Isolation of the Nuclear Matrix

Nuclear matrix proteins were isolated by the procedure of Berezney and Coffey (13). Six rat livers were homogenized with 10 strokes of a Teflon homogenizer in 1/4 volume of TM buffer (0.25 M sucrose, 5 mM MgCl₂, 0.05 M Tris, pH 7.4) plus freshly added 1 mM PMSF, and 1 mM sodium tetrathionate (10). The homogenate was filtered through four layers of cheesecloth, placed in cellulose nitrate tubes (half full), and underlaid with 2.4 M sucrose in TM buffer. A crude gradient was formed by stirring the interface and centrifuged at 38,000 g (avg) for 60 min in an SW27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 4°C. The nuclear pellet was resuspended in 30 ml of TM buffer and treated with DNase I (2.5 µg/ml) and RNase A (2.5 µg/ml) at 22°C for 15 min. The nuclear suspension was centrifuged at 770 g (avg) for 15 min and the pellet was washed once in TM buffer. The nuclei were stored overnight at 0°C in TM buffer, then centrifuged at 770 g for 15 min. These nuclei were resuspended in 0.2 mM MgCl₂, 10 mM Tris, pH 7.4, at a concentration of 2 mg/ml, incubated for 10 min at 0°C, and then centrifuged 770 g for 30 min. This step was repeated twice at 4 mg protein/ml. The nuclei were resuspended in 2 M NaCl, 0.2 mM MgCl₂, 10 mM Tris, pH 7.4 (same volume as the previous wash), incubated at 0°C for 10 min, and then centrifuged at 770 g for 60 min. This step was repeated twice. The nuclei were then treated with 1% Triton X-100 at 4 mg protein/ml, stirred at 0°C for 10 min, and centrifuged at 770 g for 30 min. The nuclear pellet was resuspended in TM buffer to a final concentration of 2 mg initial protein/ml and incubated for 60 min at 22°C in the presence of 5 µg/ml DNase I and 200 µg/ml RNase A. The suspension was then centrifuged at 770 g for 30 min. The nuclear matrix was washed twice with TM buffer with 770 g centrifugation for 20 min, dialyzed against distilled water and 0.01% βME, and lyophilized.

Isolation of Residual Nucleoli

After the initial mild DNase digestion step in the isolation of nuclear matrix, the nuclei were washed once in TM buffer and resuspended in 0.25 M sucrose, 10 mM Tris, pH 7.4, 1 mM CaCl₂ to a final concentration of 1 mg protein/ml. The nuclei were sonicated with 20-s bursts of a Branson Probe sonicator (model S-75, Branson Sonic Power Co., Danbury, Conn.) at setting 4 until no intact nuclei remained, as monitored by phase-contrast microscopy. The sonicate was underlaid with 0.9 M sucrose, 10 mM Tris, pH 7.4, and centrifuged at 3,000 g for 20 min. The pellet was resuspended in sucrose-Tris-Ca⁺⁺ buffer, again underlaid with the 0.9 M sucrose buffer, and re-centrifuged. The nucleoli were stored overnight in TM buffer with 1 mM PMSF and 1 mM sodium tetrathionate at 0°C. The residual nucleoli were then treated in the same manner as for the nuclear matrix beginning with the TM buffer washes except that all nucleolar fractions were centrifuged at 3,000 g for 60 min.

Isolation of the Nuclear Pore Complex

Isolation of the nuclear pore complex was by the procedure of Aaronson and Blobel (1). Isolated rat liver nuclei at a concentration of 6×10^6 /ml (~2 mg protein/ml) were incubated at room temperature for 15 min in 8 mM Tris, pH 8.5, 0.1 mM MgCl₂, 11 mM β ME, 0.25 M sucrose, 1 μ g/ml DNase I, and incubated at room temperature for 20 min. 2 vol of cold distilled water was added and the suspension centrifuged at 1,000 g for 20 min at 4°C. The pellet was resuspended in 12 ml of cold 0.25 M sucrose, 50 mM Tris, pH 7.5, 25 mM KCl, 5 mM MgCl₂ to which 3 ml of 10% Triton X-100 (vol/vol) was added. This was incubated at 0°C for 10 min then centrifuged at 1,000 g for 15 min. The pellet was resuspended in cold 0.25 M sucrose, 50 mM Tris, pH 7.5, 25 mM KCl, 5 mM MgCl₂, and 1 M MgCl₂ added to give a final concentration of 0.3 mM MgCl₂. After centrifugation at 1,000 g for 15 min, the pellet was dialyzed against distilled water and 0.01% β ME and lyophilized.

Isolation of hnRNP Particles

The hnRNP particles were isolated by the procedure of Samarina et al. (88). Isolated nuclei were washed three times in STM-7 (10 mM Tris, pH 7, 0.15 M NaCl, 1 mM MgCl₂, 5 mM β ME, 0.1 mM PMSF) followed by four washes in STM-8 (pH adjusted to 8). The combined supernates were clarified by centrifugation at 2,000 g for 10 min and centrifuged through a 15-30% sucrose gradient for 15 h at 23,000 rpm in an SW27 rotor. The 30S peak was dialyzed against β ME and lyophilized.

Preparation of Chromatin

Chromatin was prepared by the procedure of Bonner et al. (18) from purified nuclei. Nuclei were washed twice with saline-EDTA buffer, then five times with 10 mM Tris. All buffers contained 5 mM β ME and 0.1 mM PMSF. Final pellets were resuspended in Tris buffer and purified through 1.7 M sucrose. Purified chromatin was dialyzed overnight against Tris buffer, sheared in a VirTis 65 (VirTis Co., Inc., Gardiner, N.Y.), and unsheared material was pelleted at 10,000 rpm for 10 min. Chromatin prepared this way had an A360/A260 of 0.04 and a protein to DNA ratio of 1.8. The final chromatin was centrifuged at 100,000 g for 18 h in the presence of 1% SDS to pellet the DNA. Analysis of the supernate revealed that > 90% of the protein is removed by this procedure. This supernate was then treated in the same way as the washes regarding dialysis, ethanol treatment, and lyophilization.

Preparation of Samples for Lyophilization

After the 100,000 g centrifugations of cytoplasm and SE and Tris nuclear washes (see above), fresh PMSF was added to the supernates, they were allowed to warm slightly at room temperature, then 10% SDS was added to a final concentration of 1%. The samples were dialyzed against two changes of 5 mM β ME overnight, then dialyzed against two changes of 95% EtOH. This step solubilizes free SDS and allows it to be removed from the precipitated SDS-protein complexes by centrifugation at 10,000 rpm for 15 min in the HB-4 rotor. Protein pellets were resuspended in a minimal volume (~2 ml) of 5 mM β ME and lyophilized.

Sample Preparation for Isofocusing

Lyophilized samples were resuspended in a minimal volume of 2% SDS, 5 mM EDTA, and 5% β ME in a Corning 15-ml plastic conical test tube (Corning Glass Works, Science Products Div., Corning, N.Y.). The sample was bath sonicated for several seconds to dissolve or at least totally disperse the material. We find the plastic tubes much superior to glass for this purpose because of the improved transmission of ultrasonic vibration during sonication. This was followed by boiling for no more than 1 min. We have found that longer times increased the risk of artifacts in the isofocusing dimension (see also reference 99). The samples were then allowed to cool to room temperature, upon which time urea was added at a concentration of 0.8 mg/ μ l of original SDS sample buffer volume. As the result of a slight volume loss on boiling, this represents an effective concentration of > 9 M urea. The protein concentration was determined on a 5-10- μ l aliquot of this material and then pH 3-10 ampholytes were added to 4% (absolute ampholyte concentration relative to total sample volume) and NP40 was added such that the final concentration of detergent was 10% of the original SDS volume. Samples were applied to the gels in volumes not exceeding 150 μ l. Larger volumes tended to cause a loss of resolution and a shifting of the pH gradient toward the acidic end caused by the relatively large amount of SDS in the gel. Samples were overlayed with an agarose-urea solution (1% agarose, 0.5 M urea) to protect against the cathodic buffer, particularly the precipitated CaCO₃ (see below) which settles onto the surface of the agarose with time.

Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed essentially according to O'Farrell (78) with the following modifications: Isofocusing was carried out in 19-cm gels of 3.2-mm internal diameter using the following solution to pour four gels: 1 ml of acrylamide (28.38% acrylamide, 1.62% bis), 2 ml of 10% NP40, 2.5 ml H₂O, 5.6 g of ultrapure urea, 600 μ l of Biolyte ampholyte (3-10, 100 μ l; 6-8, 200 μ l; 5-7, 300 μ l). Catalysts used were 100 μ l of 10% ammonium persulfate, 8 μ l of *N,N,N',N'*-tetramethylethylenediamine (TEMED), and 5 μ l of riboflavin (0.5 mg/ml). Gels were allowed to polymerize under ultraviolet light for a minimum of 4 h and usually overnight. Samples were loaded onto the gels (see below) and run in a Savant high-voltage electrophoresis unit (Savant Instruments, Inc., Hicksville, N.Y.) with the accompanying lower chamber being replaced by a 25-cm high Lucite reservoir to accommodate the longer gels. Electrophoresis was carried out with a Hoefer PS1200 DC programmable power supply (Hoefer Scientific Instruments, San Francisco, Calif.). Electrode buffers were 0.01 M phosphoric acid (anode) and 0.04 M NaOH, 0.02 M Ca(OH)₂ (cathode). The Ca(OH)₂ removes dissolved CO₂ by forming insoluble CaCO₃; we find that this adds stability and reduces variation of the pH gradients in different runs. Runs were programmed at a constant current of 0.5 mA/tube until the voltage reached a present value of 800 V. Subsequent electrophoresis was then at this constant voltage. Gels were run for 17 h, and then hyperfocused for 1 h at 1,000 V. pH measurements were made at 1-cm intervals directly on the extruded gels using a Sensorex type 450C flatbed combination electrode (Sensorex, Westminster, Calif.).

The second dimension was run according to O'Farrell (78) employing the Laemmli (65) system except that four isofocusing (IF) gels were run simultaneously on one continuous slab gel of 20- × 86-cm dimensions. Individual IF gels were anchored into place with a 1% agarose solution containing 0.5 M urea and 0.005% bromophenol blue tracking dye. After constant voltage electrophoresis at 60 V for ~1.5 h through the stacking gel (4.75% acrylamide) and 120 V for 7-8 h through the resolving gel (10% acrylamide), the gel was then sliced into four 20- × 20-cm slabs and stained overnight in 0.05% coomassie blue R in 10% acetic acid, 25% isopropanol. Destaining was accomplished by first placing gels in 10% acetic acid, 25% isopropanol for 1-2 h followed by several changes with 10% acetic acid. They were photographed the following day, with the orientation such that the basic end is to the left and the acidic end is to the right.

Molecular weight values were assigned using the log molecular weight vs. R_f relationship described by Shapiro et al. (92). The molecular weight of any given protein was determined from a comparison of its mobility with that of standards run in the same gel in both dimensions.

Nonequilibrium Gel Electrophoresis

To examine the more basic proteins in these washes the nonequilibrium pH gradient electrophoresis (NEPHGE) system of O'Farrell et al. (79) was used and modified to accommodate our system. The constitution of the IF gels was identical to the equilibrium method, with two exceptions. Only pH 3-10 ampholytes were used (500 μ l/solution for four gels) and the catalysts were increased in amount because of the higher proportion of basic ampholytes present in the mixture. Ammonium persulfate was increased to 20 μ l and TEMED to 14 μ l. The amount of riboflavin remained unchanged. Samples were again overlaid with 100 μ l of 1% agarose in 0.5 M urea, but in this case the agarose was layered onto 25 μ l of sample overlay containing 8 M urea, 5% NP40, and 1% 3-10 ampholyte. Electrophoresis was carried out with reversed polarity as suggested by O'Farrell and run at 400 V for 11 h. Subsequent handling of the gels and SDS gel electrophoresis was identical to that employed in the equilibrium system.

Protein Determination

Protein concentrations employed in the gel electrophoresis were determined by a rapid TCA-turbidity procedure previously described (36). Quantitative evaluation of the proteins of the nuclei, nuclear washes, etc. was obtained by dialyzing 0.5- to 1.0-ml samples overnight against 1 N NaOH and assaying by the method of Lowry et al (72).

Chemicals

Protein standards phosphorylase *a* (P-1261), 3-phosphoglycerate kinase (P-7634), carbonic anhydrase (C-7500), and soybean trypsin inhibitor (T-9003) were purchased from Sigma, Chemical Co. (St. Louis, Mo.), as were ammonium persulfate, glycine, Na₂EDTA, PMSF, and SDS. NP-40 was obtained from Particle Data Inc. (Elmhurst, Ill.), and riboflavin was purchased from Eastman Kodak Co. (Rochester, N.Y.). Bovine serum albumin (9385) was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio). Rat skeletal myofibril, containing actin as the principal component, was prepared according to the procedure

of Etlinger and Fischman (42). Ultrapure urea was purchased from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N.Y.), acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED), and ampholytes from Bio-Rad Laboratories (Richmond, Calif.).

RESULTS

Before an accurate and reproducible comparison among gels of widely differing protein constitution could be attempted, we needed to devise a reference system whereby the precise location of polypeptide spots in different gels could be compared. We arrived at such a system by choosing protein standards that provided an effective "compartmentalization" of each gel and are commercially available or easily prepared. This was important, because one of our goals was to establish a system that could serve as a useful guide in comparing specific NHP among different laboratories.

Fig. 1 represents the kind of partitioning that can be obtained with the six standards chosen. Drawing an *x*- and *y*-intercept through the standards divides the gel into 36 sections. The observed isoelectric points are relatively meaningless because pH measurements in urea tend to be higher than in its absence: this is caused by the reduction of hydrogen ion activity (22) and possibly direct effects on the pH electrode itself (96). Aberrant isoelectric points determined in urea can also result from the denaturation of the protein (96).

Because the absolute isoelectric points of the standards employed were mostly determined in the absence of urea, the position of a given polypeptide between two *x*-intercepts can give an estimate of its true isoelectric point. However, because we were more concerned about the positions of the spots relative to the internal standards, subsequent gels are presented with measured pH gradients omitted. Further details on the use of internal standards is presented elsewhere (34).

Electrophoresis of Rat Liver Cytoplasm and Nuclei

Fig. 2 is a representative example of two-dimensional gel electrophoresis of a preparation of rat liver cytoplasm. The livers were perfused with saline before excision from the animal to remove contaminating serum proteins. The absence of albumin in Fig. 2 attests to this. We have not made any attempt to identify the cytoplasmic proteins except those that are also present in the nucleus and/or nuclear washes. Those cytoplasmic polypeptides that are also present in the nuclear washes have been circled and assigned a molecular weight. Although we term these proteins "cytoplasmic", it is possible that some might be nucleoplasmic proteins that have leaked into the cytoplasm during homogenization. In this kind of study there is no way of ascertaining with any certainty whether or not this has occurred. However, the easily extracted saline-EDTA wash proteins constitute only 15% of the total nuclear proteins, whereas the cytoplasmic proteins constitute the majority of the soluble cellular proteins. Therefore, for a nucleoplasmic protein to become a major cytoplasmic species observed in our gels and not be present in relatively greater amounts in the nucleoplasm seems unlikely (cf., Figs. 2 and 4).

One striking observation from the comparison of the cytoplasmic proteins in Fig. 2 with those of the whole nuclei and nuclear washes (Figs. 3-6) is that the vast majority of the cytoplasmic species are more basic than bovine serum albumin (intrinsic pI = 4.9) and certainly more basic than actin, whereas a much higher percentage of the nuclear proteins have isoelectric points nearer to or more acidic than that of actin. Of the

more than 350 polypeptides observed in the cytoplasm, only five are also present in all of the nuclear washes and final chromatin. These are circled and single-hatched in Fig. 2 (*C + N*). They are a 75,000-mol wt polypeptide between actin and soybean trypsin inhibitor (*A-S*), two groups of 68,000-dalton polypeptides in sectors *C-B* and *B-A*, a 57,000-dalton polypeptide at the *B* intercept, and two near actin at 43,000 daltons. All other designated spots in Fig. 2 are gradually diminished and ultimately removed from the nucleus by the first Tris wash.

Fig. 3 illustrates the gel pattern of unwashed rat liver nuclei. The fact that the gel of unwashed nuclei appears to be relatively free of cytoplasmic contamination, although the saline-EDTA (SE) washes (see Fig. 4) show many cytoplasmic proteins, indicates that the degree of cytoplasmic contamination of the unwashed nuclei is obscured by the much greater abundance of specific nuclear proteins. The five ubiquitous proteins mentioned above are single-hatched (*C + N*). An additional set of polypeptides found only in the nuclear washes or final chromatin and not in the cytoplasmic fraction are double-hatched (*N*). These include a polypeptide at 94,000 daltons, a group at 54,000-55,000 daltons, and two at 25,000 and 22,500 daltons between *A* and *S*.

Most of the other proteins in Fig. 3, which have been circled with dotted lines and assigned molecular weight values, are tightly associated with the washed chromatin. For the following reasons, the 43,000-dalton protein near *A* appears to be actin: (a) It is found in both the cytoplasm and nucleus (68); (b) using two-dimensional gel electrophoresis, Garrels and Gibson (47) found nonmuscle actin to be slightly more basic than muscle actin, similar to protein 43; and (c) we find that rat liver nuclear actin isolated by DNase I affinity chromatography by the method of Lazarides and Lindberg (67) co-migrates with protein 43 on two-dimensional gels.

In Fig. 3 the group of proteins at the far left of the gel at molecular weights of 34,000 and 36,000 are hnRNA-associated proteins. They are more clearly evident in NEPHGE of Tris washes (Figs. 6 and 9).

Electrophoresis of Nuclear Washes

The percent of the total nuclear protein that was solubilized in the SE and Tris washes is shown in Table I. Approximately 25% of the total protein associated with unwashed nuclei is removed by these washes.

Figs. 4-6 show the distribution of proteins soluble in the SE and Tris washes of rat liver nuclei. Most of the contaminating cytoplasmic proteins are removed from the nucleus in the first SE wash. They disappear altogether by the first Tris wash (not shown). The Tris washes (Fig. 5) also show (single-hatched) the ubiquitous 75,000-, 68,000-, 57,000-, and 43,000-dalton proteins. Also, note the appearance of the 62,000-mol wt Q1 and Q2 proteins, which are associated with the nuclear matrix (Fig. 7). Only Q2 is apparent in the SE-1 wash. Its counterpart, Q1, begins to appear in SE-2 washes (not shown) and both Q1 and Q2 are major constituents of the Tris washes and final nuclear pellet.

In the first and second SE washes there is a 25,000-mol wt protein streaked near the basic end of gels. This protein is a major protein of the SE-1 wash of mouse liver nuclei (38) and has been purified to homogeneity in our laboratory (Conner and Comings, manuscript in preparation). It is closely related to the high mobility group (HMG) proteins first reported by Goodwin et al. (51) and Goodwin and Johns (50).

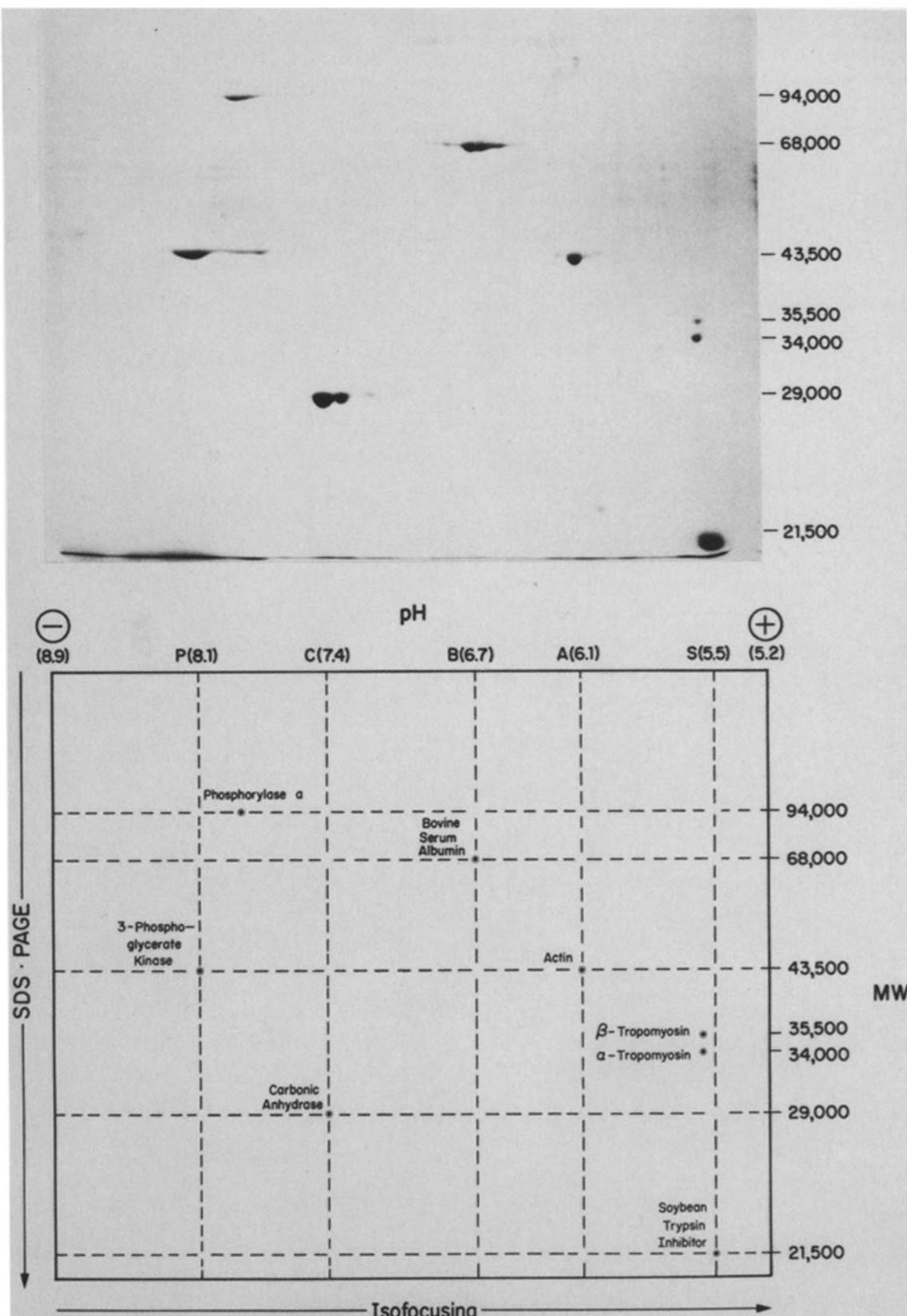


FIGURE 1 Standard two-dimensional gel electrophoresis of protein standards. A lyophilized mixture of standards containing the following quantities of protein was electrophoresed as described in Materials and Methods: rat skeletal muscle myofibril, 30 µg; phosphorylase α , 14 µg; BSA, 5 µg; 3-phosphoglycerate kinase, 8 µg; carbonic anhydrase, 10 µg; and soybean trypsin inhibitor, 8 µg. The diagram below the photograph of the gel depicts the grid reference pattern obtained by drawing horizontal and vertical lines through the indicated standards. The letters *P*, *C*, *B*, *A*, and *S* at the top of the diagram designate the coordinates of 3-phosphoglycerate kinase, carbonic anhydrase, BSA, actin and soybean trypsin inhibitor, respectively. Numbers in parentheses indicate pH values at these coordinates derived from the pH gradient measured in the first dimension (isofocusing) gel. In all gels, vertical lines drawn through 3-phosphoglycerate kinase, carbonic anhydrase, and BSA are drawn through the most basic or "parent" spot. All gels are photographed with the basic end to the left and the acidic end to the right. The second dimension was run in 10% acrylamide.

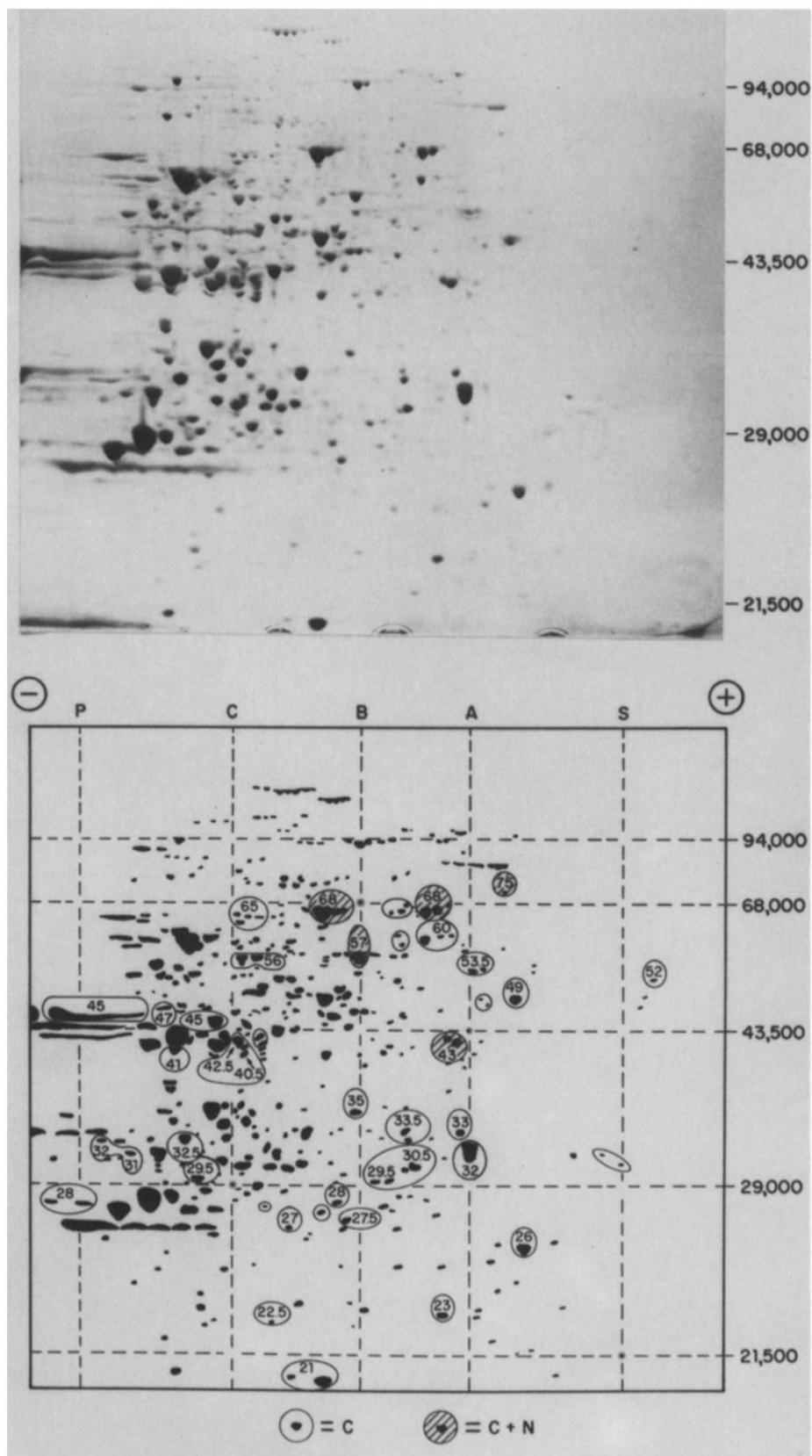


FIGURE 2 Standard two-dimensional gel electrophoresis of rat liver cytoplasm. 400 µg of cytoplasmic proteins was electrophoresed as described in Materials and Methods, with 10% acrylamide in the second dimension. The grid pattern was obtained from a simultaneously run duplicate gel containing the protein standard seen in Fig. 1. C, cytoplasmic proteins. C+N, proteins found in the cytoplasm, nuclear washes, and final chromatin pellet.

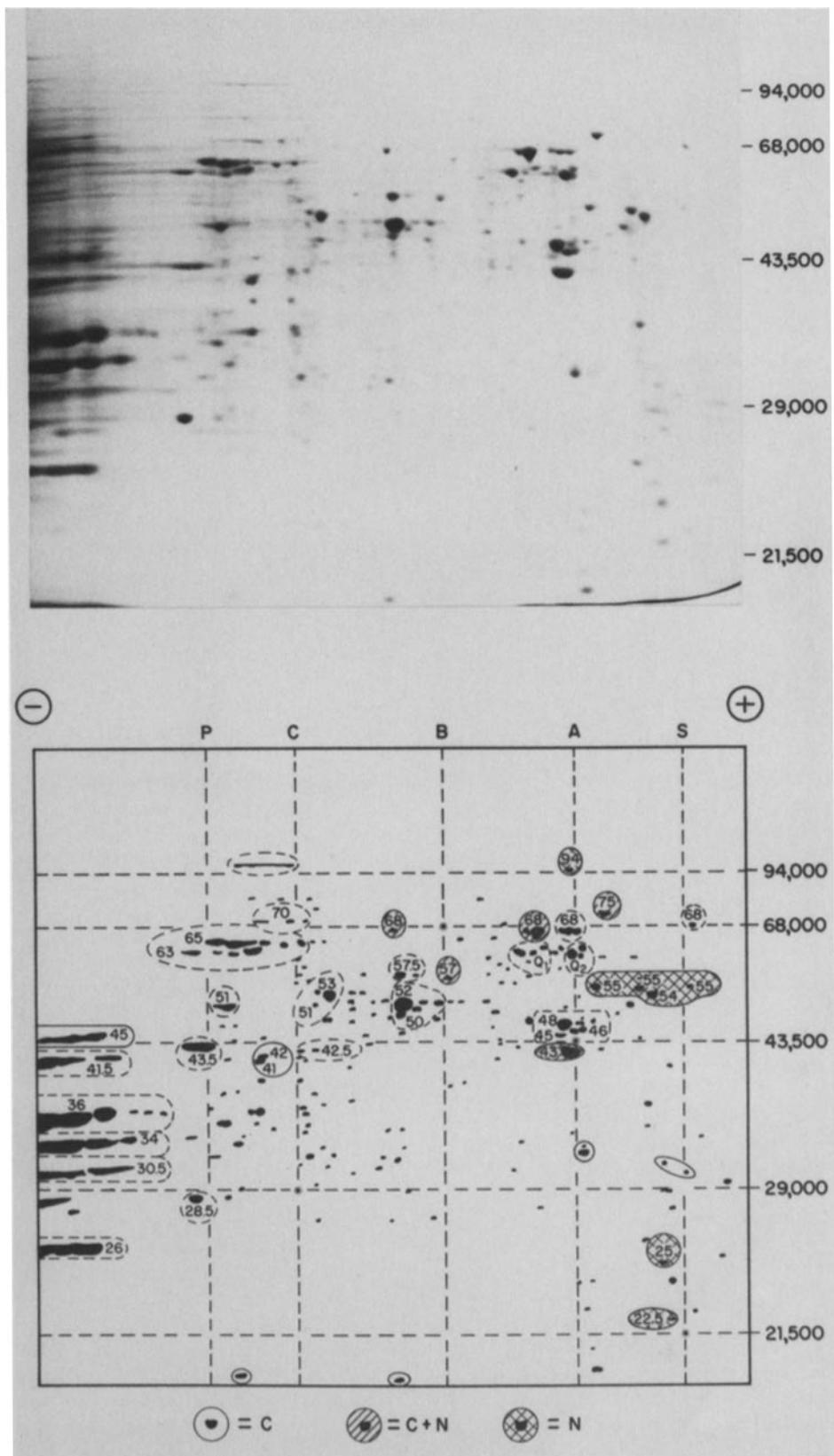


FIGURE 3 Standard two-dimensional gel electrophoresis of unwashed rat liver nuclei. 1,200 µg of nuclear proteins was electrophoresed under the same conditions as those described in Figs. 1 and 2. C, cytoplasmic proteins; C+N, proteins found in the cytoplasm, all nuclear washes, and final chromatin pellet; N, proteins not found in cytoplasm, but which are present in all nuclear washes. Spots circled with dotted lines and assigned molecular weight values indicate hnRNP, nuclear matrix proteins, and other proteins that are prominent in gels of whole nuclei.

TABLE I
Protein Content of Nuclear Washes

Sample	Protein*	Total‡
	mg	%
Unwashed nuclei	174.7 ± 10.0	100
First saline-EDTA wash	25.2 ± 0.8	14.4
Second saline-EDTA wash	11.4 ± 0.4	6.5
First Tris wash	4.4 ± 0.4	2.5
Second Tris wash	3.1 ± 0.5	1.8
Final nuclear pellet	124.8 ± 4.9	71.4

* Results are expressed as the mean and standard error of the mean for six experiments and reflect yields from an average of 7.8×10^9 nuclei representing ~60 g wet weight of liver.

‡ The total percentage of protein in the washes and final pellet is 96.6 rather than 100% because of the fact that the wash recoveries were determined on the clarified 100,000-g supernates, and some broken nuclei and nuclear membranes were pelleted during this step.

The polypeptides of the first Tris wash (not shown) were identical to those of the second Tris wash seen in Fig. 5, and are strikingly different from those of the SE wash in Fig. 4. There are fewer proteins, and those that are present tend to mimic more closely the pattern observed in gels of chromatin (Figs. 10 and 11), with the exception of a few major groups. Also, the predominance of the more acidic proteins is apparent when comparing this pattern with that seen, for example, in Fig. 2. The single-hatched ubiquitous group, that is, those proteins of molecular weight 75,000, 68,000, 57,000, and 43,000 daltons, is quite prominent, particularly the 68,000-mol wt proteins at *B-A* and the 43,000-dalton protein. The nuclear proteins at 94,000, 54,000-55,000, 25,000, 22,500, and 20,500 daltons are very pronounced in this wash, with the 25,000-dalton protein being particularly striking. Q1 and Q2 are now major species, and one can also see quite clearly the emergence of proteins associated with the nuclear matrix and/or hnRNA. These include two groups in the 62,000-68,000-mol wt range (quadruple spots 1 and 2 [Q1, Q2], 68,000 *B-A*, and 63,000 and 65,000 *P-C*), two spots at 57,000 *C-B*, the group of three to four proteins at 54,000-55,000 *A-S*, those at 52,000 *B* and 48,000 *A*, and the groups spread out at 42,000 and 41,000 *C-B*.

The Tris washes also reveal a very prominent group of possibly related species in the 31,000-33,000 mol wt range at *A* which are not matrix proteins nor are they associated with hnRNP particles.

The 34,000- and 36,000-dalton set of proteins at the far left of the gel in Fig. 5 are only partially resolved in the isofocusing dimension. This particular situation pointed out the need for greater resolving power in the basic region of these gels to analyze such patterns. Because many of the hnRNP particle proteins have been reported to be quite basic (15, 58, 94), we examined the washes using the NEPHGE system of O'Farrell et al. (79). Fig. 6 shows the same Tris wash as that seen in Fig. 5, but in this case run by NEPHGE. Although almost all of the spots seen in Fig. 5 can be located in the NEPHGE gel, the acid half of the gel has been compressed, reducing the resolution of the more acidic proteins. However, the resolution of the basic end has been increased, and proteins are observed which had not entered the standard gel. The 34,000- and 36,000-mol wt proteins are now clearly separated into two major series of spots extending in the acid direction from hn (used as an internal marker in the NEPHGE system) as well as numerous less prominent species. In this gel, those which are hnRNA-associated proteins (see below) have been circled with dotted

lines and numbered I and II for easier identification. Those labeled III, IV, and V, or simply circled, are nuclear matrix-associated proteins or proteins very prominent in the Tris nuclear washes. As can be seen Tris wash proteins are predominantly hnRNP or nuclear matrix proteins.

Nuclear Matrix Proteins

Electrophoresis of the nuclear matrix proteins using the NEPHGE technique is shown in Figure 7. As with one-dimensional gel electrophoresis, a major set of proteins occurs between 65,000 and 75,000 daltons. However, this two-dimensional gel demonstrates that the complexity of these proteins is far greater than previously indicated by SDS gel electrophoresis (9, 11, 13, 32). Of this group there are three subsets: an acidic set (Q1, Q2, and 68 1-5, of which only isomers 2, 4, and 5 are visible in NEPHGE gels), isofocusing near actin, an intermediate set (65 and 70) isofocusing near carbonic anhydrase, and a basic set (69 and 73) isofocusing near hn.

An interesting configuration of spots, pointed out earlier, is Q1 and Q2 represented by two very similar sets of four spots each (see also Figs. 10 and 11). Our experience with the mutant brain protein P1 Duarte (28, 33) suggests that all the polypeptides of such a cluster are coded for by the same gene. An intriguing possibility is that Q1 and Q2 are produced by separate single genes that arose by gene duplication. Another example of a conceivable gene duplication event is the set of polypeptides at 34,000 and 36,000 daltons, which are hnRNA-associated species (Fig. 9). A simple deletion of the DNA coding for 18 amino acids from a duplicate copy of the gene for hnRNA polypeptide 36 could result in a lower molecular weight species with a slight shift in isoelectric point (for example, polypeptide 34). Other similar patterns present additional instances, but rigorous biochemical studies obviously are necessary to confirm the possibility that many of the NHP are present in duplicate sets due to an ancient tetraploid event (80).

Another prominent set of proteins of the nuclear matrix are species at 70,000 and 65,000 daltons. Both have multiple-charge isomers (54). Extensive experience with various methods of protein preparation indicate these charge isomers are the result of posttranscriptional modifications and are not artifacts of the procedure (see also reference 5).

Of the lower molecular weight proteins, the 43-47-48 complex in the region of actin, and the 50-52 complex close to the isoelectric point of bovine serum albumin (BSA), are the most prominent. As mentioned earlier, protein 43 is probably actin. The most prominent basic polypeptides are the 36 set which are hnRNA-associated proteins (see below).

Table II shows the approximate quantitation of the percentage of the different polypeptides in the nuclear fractions. An underlined value means that the polypeptide is most prominent in that particular nuclear subfraction. From this, we can see that matrix proteins 41, 41 hn-C (between hn and carbonic anhydrase), 38, 37.5, 36, and 34 are derived from the hnRNP and that such proteins constitute 35% of the nuclear matrix proteins. Proteins 68, 53, 47, and 43 are from the nuclear pore-lamina complex and constitute 5.6% of the matrix proteins. Protein 33 is from the nucleolar matrix and constitutes 3.7% of the matrix proteins. These three sources constitute 45% of the nuclear matrix proteins. The major remaining proteins are 73, 70, 69, 67, 65, 52, and 50, and these presumably constitute the intranuclear matrix.

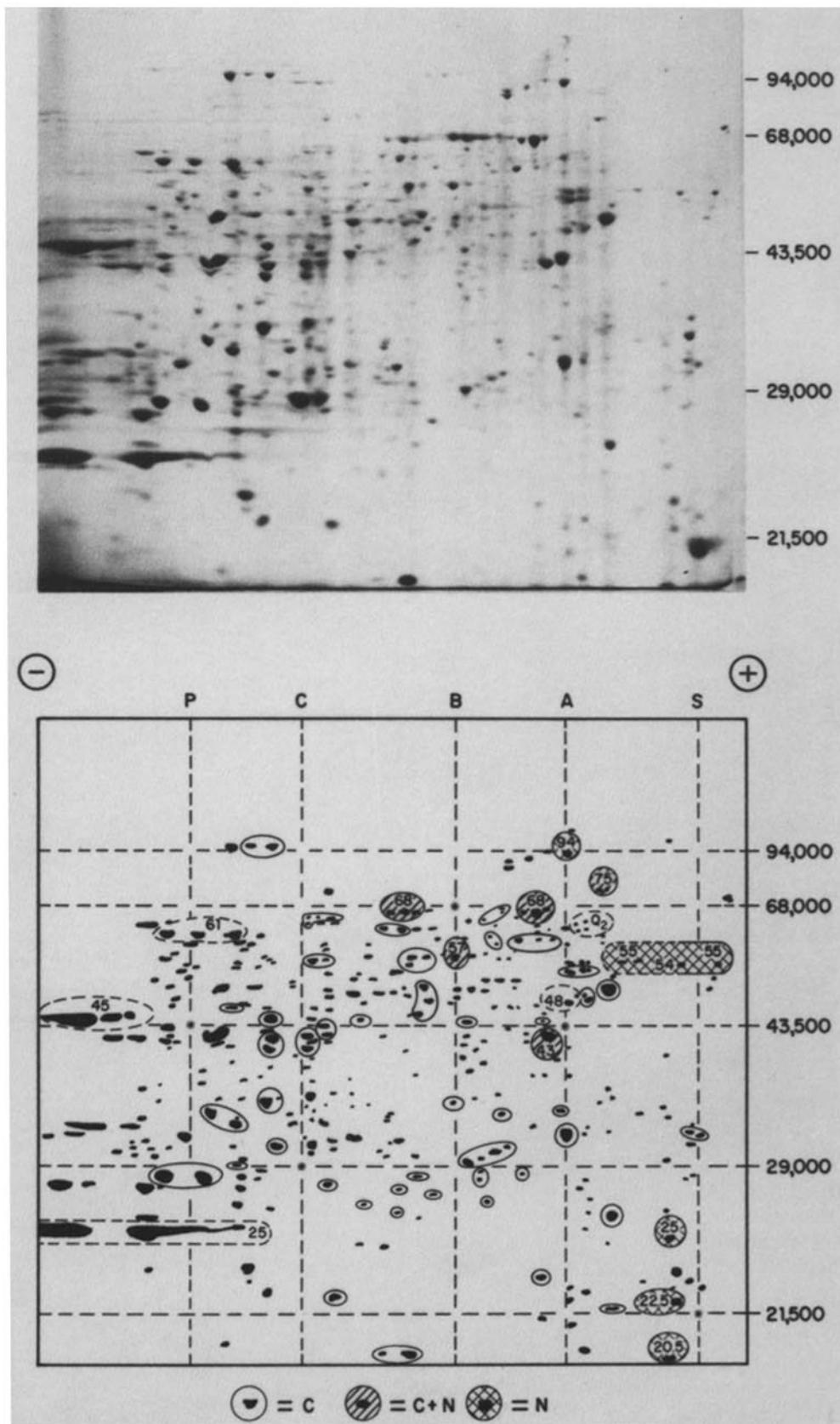


FIGURE 4 Standard two-dimensional gel electrophoresis of the first saline-EDTA wash proteins. 800 µg of protein from the first SE wash of rat liver nuclei was electrophoresed under the same conditions as those described in Figs. 1-3. See Fig. 3 for details. This gel contains the protein standards shown in Fig. 1, but these spots have been omitted in the diagram for clarity.

Nuclear Membrane-Lamina Proteins

Electron microscopy of the nuclear membrane fraction showed the inner nuclear membrane and scattered nuclear pores associated with a more feather-like material (not shown).

NEPHGE of this material is shown in Fig. 8. Here the major polypeptide is isomer 4 of the 68,000-m_r wt polypeptide. Also very prominent was 43 (actin), the 47 set and, to a lesser degree, the 50-52 set. These are presumably the 43,000-, 47,000-, 53,000-, and 64,000- to 74,000-dalton nuclear membrane pro-

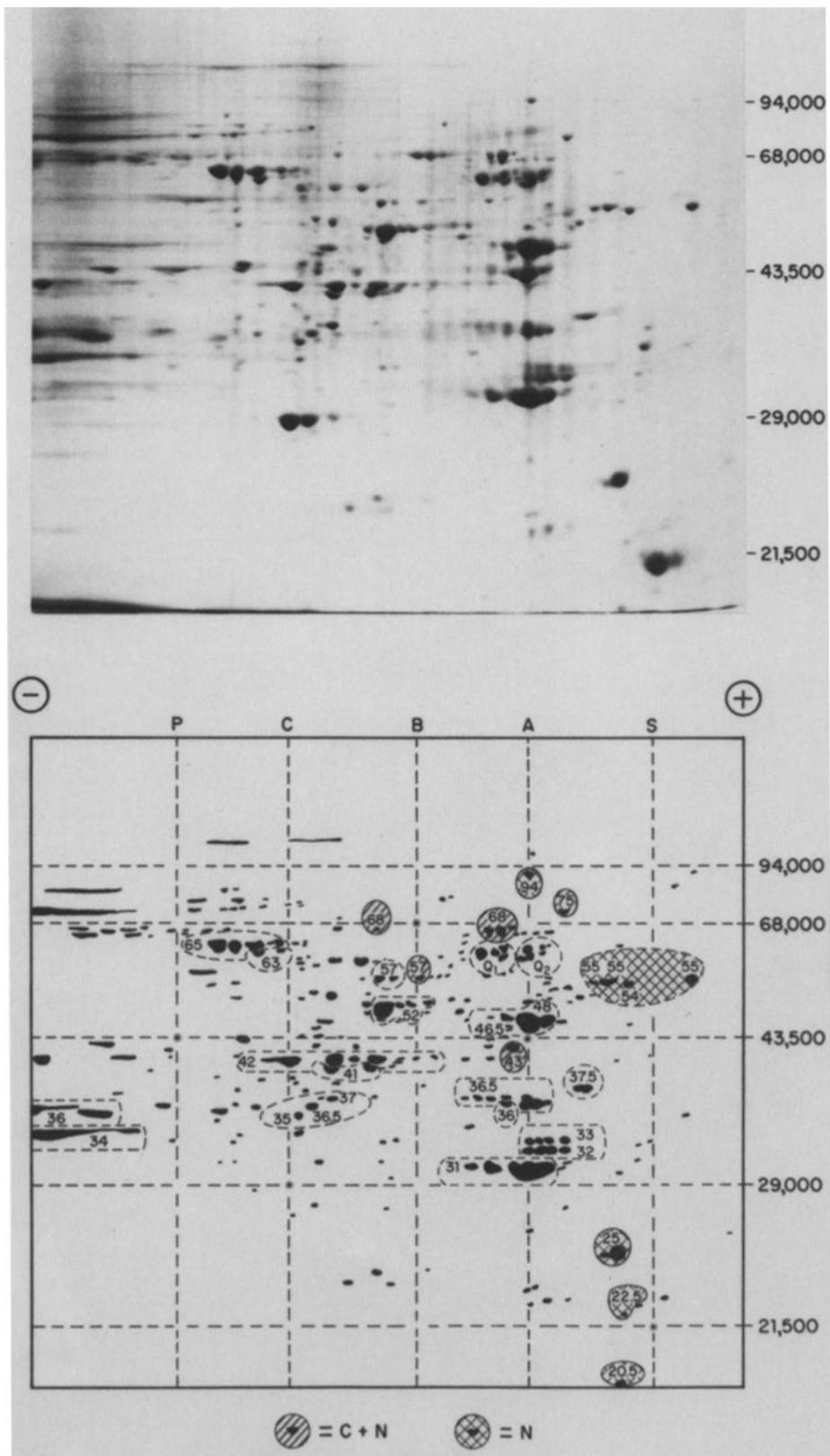


FIGURE 5 Standard two-dimensional gel electrophoresis of the second tris wash proteins. Sample load was identical to that in Fig. 4. See Fig. 3 for description. Protein standards in this gel are omitted as in Fig. 4.

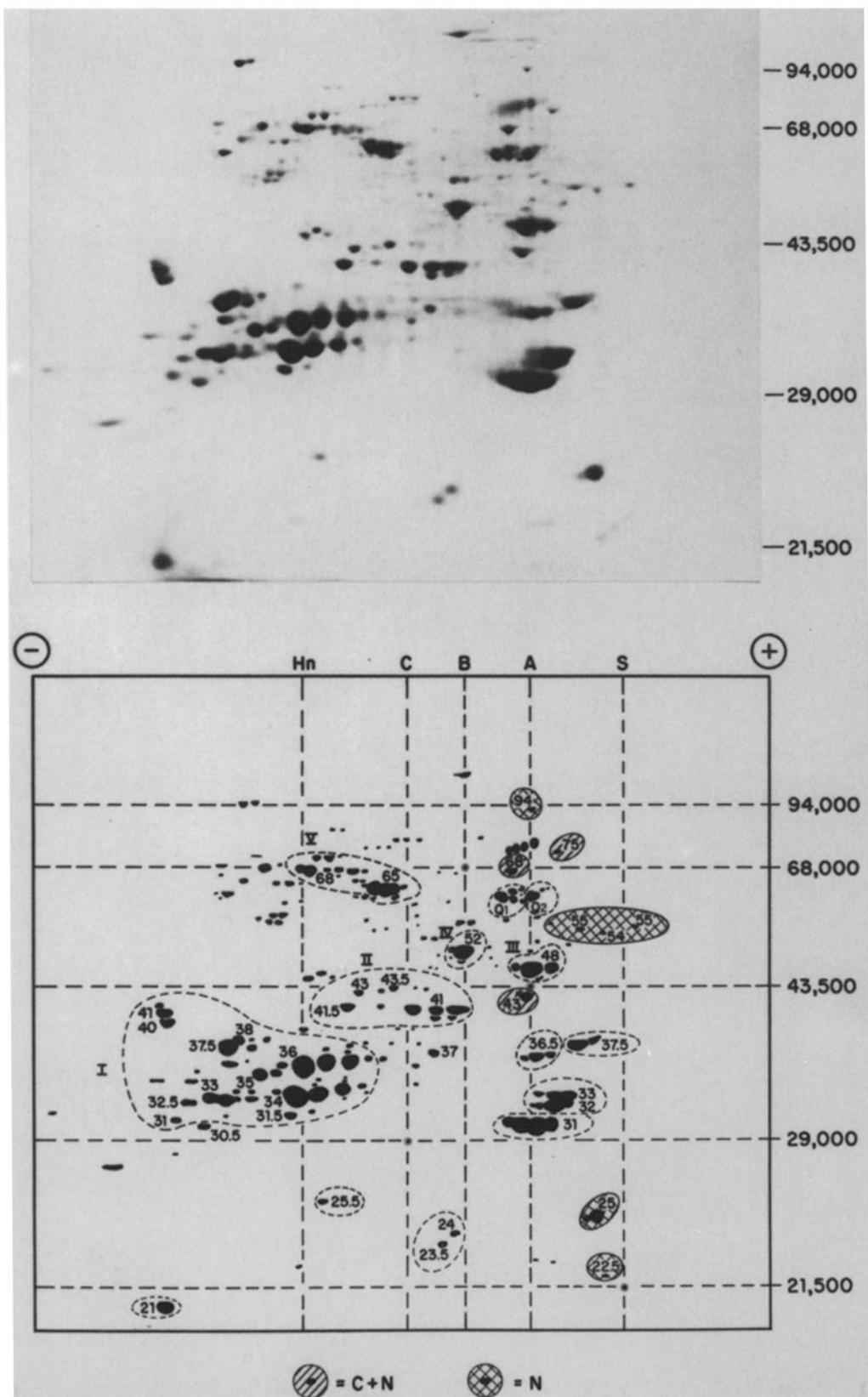


FIGURE 6 NEPHGE of second Tris wash proteins. 930 µg of sample was electrophoresed by the NEPHGE system as described in Materials and Methods. Description is the same as that in previous figures with the following exceptions. hn, representing the most basic of the 36,000-dalton series of hnRNP particle isomers, is used as an internal reference standard in this and all subsequent figures of NEPHGE gels. The groups of polypeptides circled with dotted lines and numbered I and II are associated with hnRNP particles; those labeled III, IV, and V or simply circled are nuclear matrix-associated proteins or proteins very prominent in the Tris nuclear washes.

teins described by Bornens and Kasper (20), and Kasper (59) and the 65,000- to 75,000 dalton polypeptides reported by several workers (1, 41, 49). Quantitation of these proteins (Table II) indicated that polypeptide 67 represents ~70% of the total nuclear membrane proteins. Proteins 47 and 43 constitute an additional 25%.

Nucleolar Matrix Proteins

Electron microscopy of all nuclear matrix preparations shows the presence of a nucleolar matrix (9, 11, 13, 32, 76). To determine whether these nucleolus-associated proteins are the same as those in the intranuclear matrix or are a distinct subset,

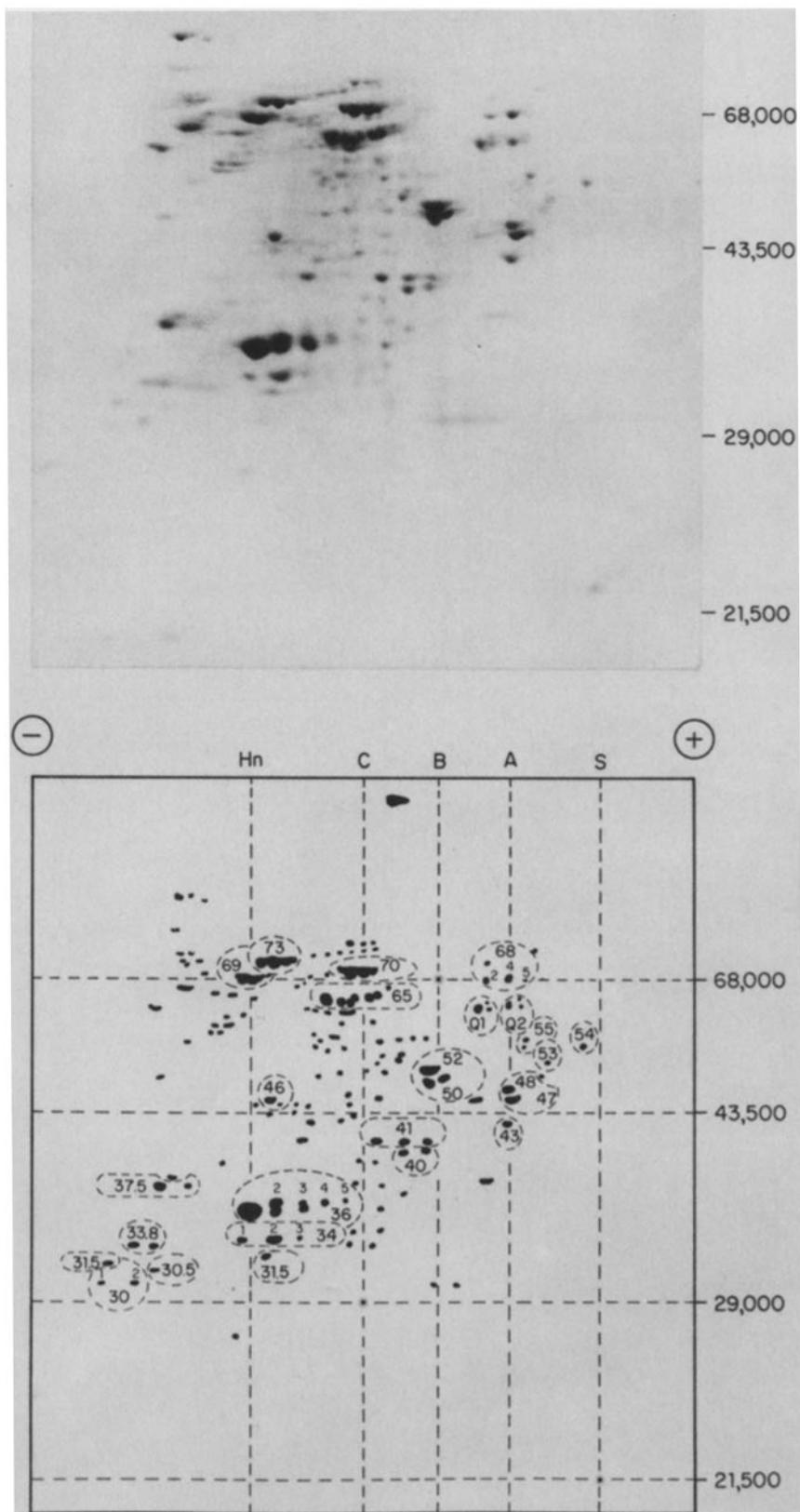


FIGURE 7 NEPHGE of rat liver nuclear matrix proteins. 1,000 µg of sample was electrophoresed by the NEPHGE system as described in Materials and Methods. For description of *hn* standard, see Fig. 6.

TABLE II
Percent of Major Polypeptides in Different Nuclear Fractions

Protein	Whole nuclei	Nuclear matrix	hnRNP	Nuclear membrane	Nucleolar matrix
94	6.4	1.4			
75	0.5	1.2			
73	1.1	8.5			
72				3.5	
70	2.1	10.9		0.4	
69	0.7	6.2			
68	1.8	2.1		67.6	0.1
67	1.9	3.2			
66					1.1
65	6.0	10.4	0.5	0.7	0.4
Q1, Q2	2.3	2.8			
64	3.5				
64 O-hn	1.2				
55		0.8		0.1	
53		0.1		0.7	
52	3.4	7.1	0.5	0.1	4.2
50		2.8		1.4	0.7
48	0.9	1.6	0.4		0.3
47	0.5	2.7		12.5	0.1
46		1.3			
44-45 hn-C	1.0				
43.5	0.5				
43	1.7	0.6	1.9	73.0	
41	6.7	2.7	14.4		
41 hn-C	2.8	1.6	2.3		
40		1.4			
38	1.8		2.9		
37.5	3.5	1.9	6.9		
36	16.1	25.7	25.5		
35	6.3		5.9		
34.5				9.6	
34	17.7	2.9	33.7	0.0	
33	5.2	3.7		66.9	
31			1.2		
30.5				7.3	
30	1.5			1.1	
29.5				3.5	
29	2.5				
28.5				2.1	
23.5				0.0	
23				0.6	
22				0.8	
21.7				1.1	

Values were obtained by averaging height and width radii for each spot and computing area. Calculations using this method on known amounts of protein show a linear relationship between area and amount of protein in a given spot.

Italicized values indicate that those polypeptides are prominent in the designated nuclear subfraction.

O-hn and hn-C represent areas of the gels between origin and hn standard, and carbonic anhydrase standards, respectively.

the nucleoli were isolated and then subjected to the same purification procedure as the nuclear matrix. Electron microscopy of this preparation (not shown) shows the pure nucleolar matrix with all of the granular elements of the nucleolus removed.

The NEPHGE of this preparation showed a small number of very basic proteins (not shown). The most prominent had a molecular weight of 33,000 daltons and constituted ~70% of the total nucleolar matrix protein.

hnRNP proteins

NEPHGE of the proteins of the hnRNP complex are shown

in Fig. 9. The predominant groups are the 34 and the 36 set. Together they constitute 60% of the total polypeptides. Next in prominence are the 41 (including 41 hn-C), 43.5, 37.5 and 35 set, constituting 17, 7, and 6%, respectively. These sets constitute over 90% of the hnRNP proteins.

Chromatin NHP

A common method of preparing the chromatin NHP is to first wash the nuclei with saline-EDTA, then with dilute Tris buffers, followed by purification through 1.7 M sucrose (18). The NHP in this chromatin is commonly assumed to be primarily involved in DNA functions. However, previous studies of ours using one-dimensional SDS gel electrophoresis had indicated that many of these so-called chromatin NHP were, in fact, nuclear matrix proteins (26, 32). It was, therefore, no startling surprise to find that two-dimensional gel electrophoresis of these chromosomal NHP also showed them to be predominantly the same proteins found in the DNA-free nuclear matrix (and hnRNP) preparations.

Fig. 10 illustrates a standard two-dimensional gel of rat liver chromatin NHP. Most of the major nuclear matrix polypeptides observed in Fig. 7 are clearly evident here with the obvious exceptions of the more basic species not resolved in this system. Those species at 43, 47-48, 52, 54, 62, 65, 68, and 70 are all associated with matrix. Q1 and Q2, at 62,000 daltons, show most clearly in this gel the characteristic quadruple-spot pattern discussed earlier. Also evident in this gel are the major nonbasic components of the hnRNP particles (Fig. 9) at 41,000 and 42,000 daltons. The ubiquitous polypeptides at 75, 68 B-A, 57 B, and 43 A are also present.

The predominance of the nuclear matrix and hnRNP polypeptides in the chromatin NHP fraction is even more clearly demonstrated in Fig. 11, which is a NEPHGE gel of the same preparation as that seen in Fig. 10, and thus gives a better perspective of the entire spectrum of chromatin-associated proteins. It was of great interest to us to discover that gels of chromatin prepared by the Bonner method (Figs. 10 and 11) were essentially identical to those of the final pellets (not shown) of the two SE and Tris buffer washes of nuclei seen in Figs. 4 and 5, even though the latter preparations did not undergo the additional five Tris washes and sucrose purification step of the Bonner procedure. Even more remarkable at first was the finding that chromatin gels looked very similar to those of untreated nuclei with respect to major components (Figs. 3 and 10), although if one considers the percentage of protein removed by the washes (20-25%) and the type of species washed free (Figs. 4 and 5) the result should not have been totally unexpected.

Quantitation of NEPHGE gels of total nuclear proteins (Table II) indicated that the only polypeptides more prominent in this fraction than in nuclear matrix or hnRNP are those species at 94, 64, and 35 O-hn (between the origin and hn marker), 41, 43.5, and 44-45 hn-C and 29 A. These constitute ~15% of the total mass. An additional 5% comes from lower frequency species more easily seen in the washes. Thus, ~80% of the mass of the total nuclear NHP is derived from the nuclear matrix and hnRNP.

DISCUSSION

To make possible the type of studies reported here a system was required which would minimize the difficulties in interpretation and analysis of the many polypeptide patterns generated. Because there are minor variations in the absolute

locations of individual spots from gel to gel (although the overall patterns are remarkably reproducible), and because pH measurements tend to be relatively inaccurate, particularly in

urea, a simple *x-y*-coordinate system using isoelectric point vs. molecular weight was insufficient. The most direct approach was to add a mixture of commonly available standards to each

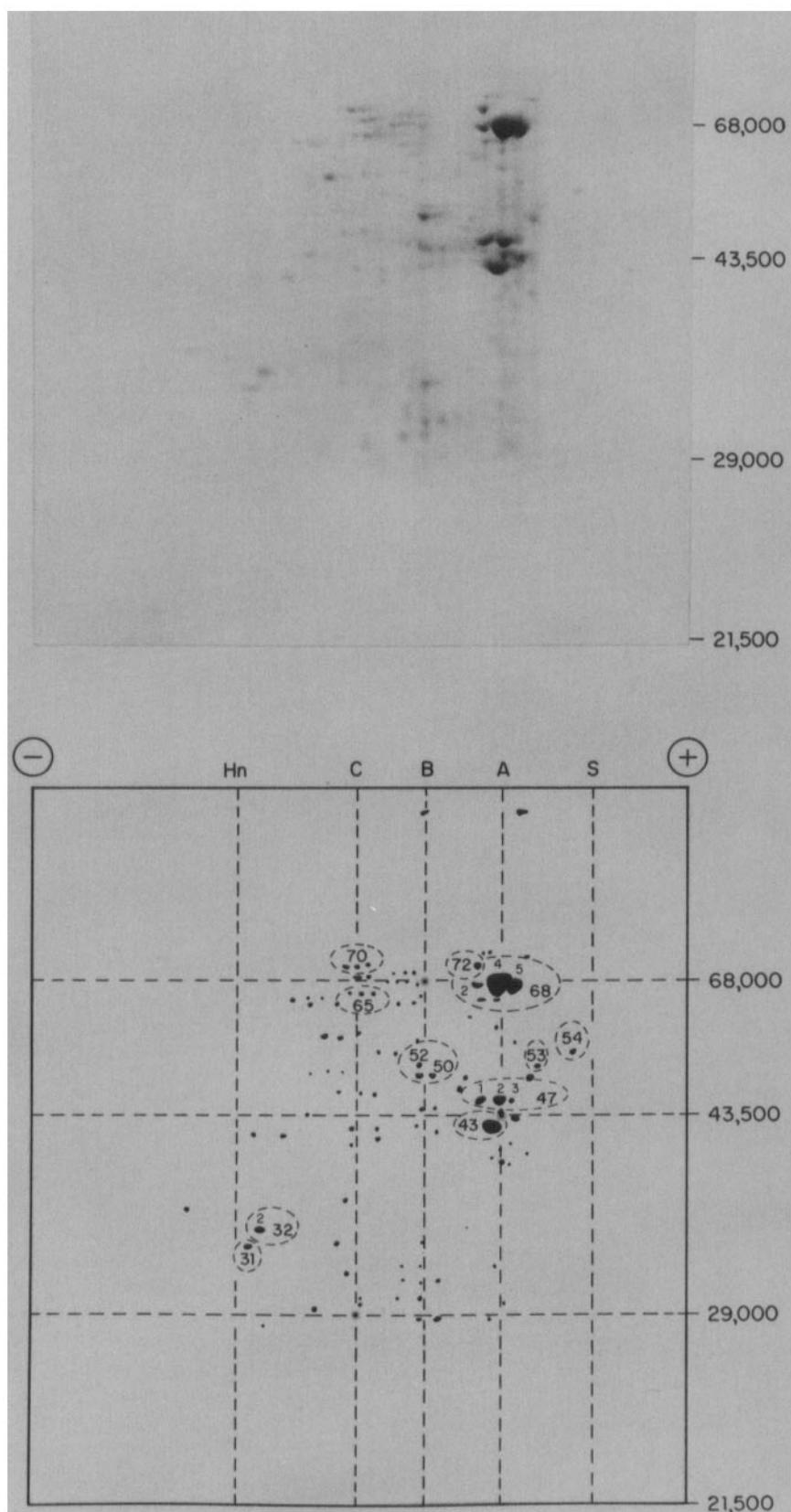


FIGURE 8 NEPHGE of rat liver nuclear membrane (lamina) proteins. 800 µg of sample was electrophoresed by the NEPHGE system as described in Materials and Methods. For description of *hn* standard, see Fig. 6.

sample, thus providing an internal reference by which gels of widely varying patterns could be compared. By choosing appropriate markers, not only were we able to "dissect" each gel into smaller, relatively equal sectors for easier analysis, but the

actual location of each spot relative to a particular standard with known physicochemical characteristics provides information pertinent to the future identification of any protein resolved by this system.

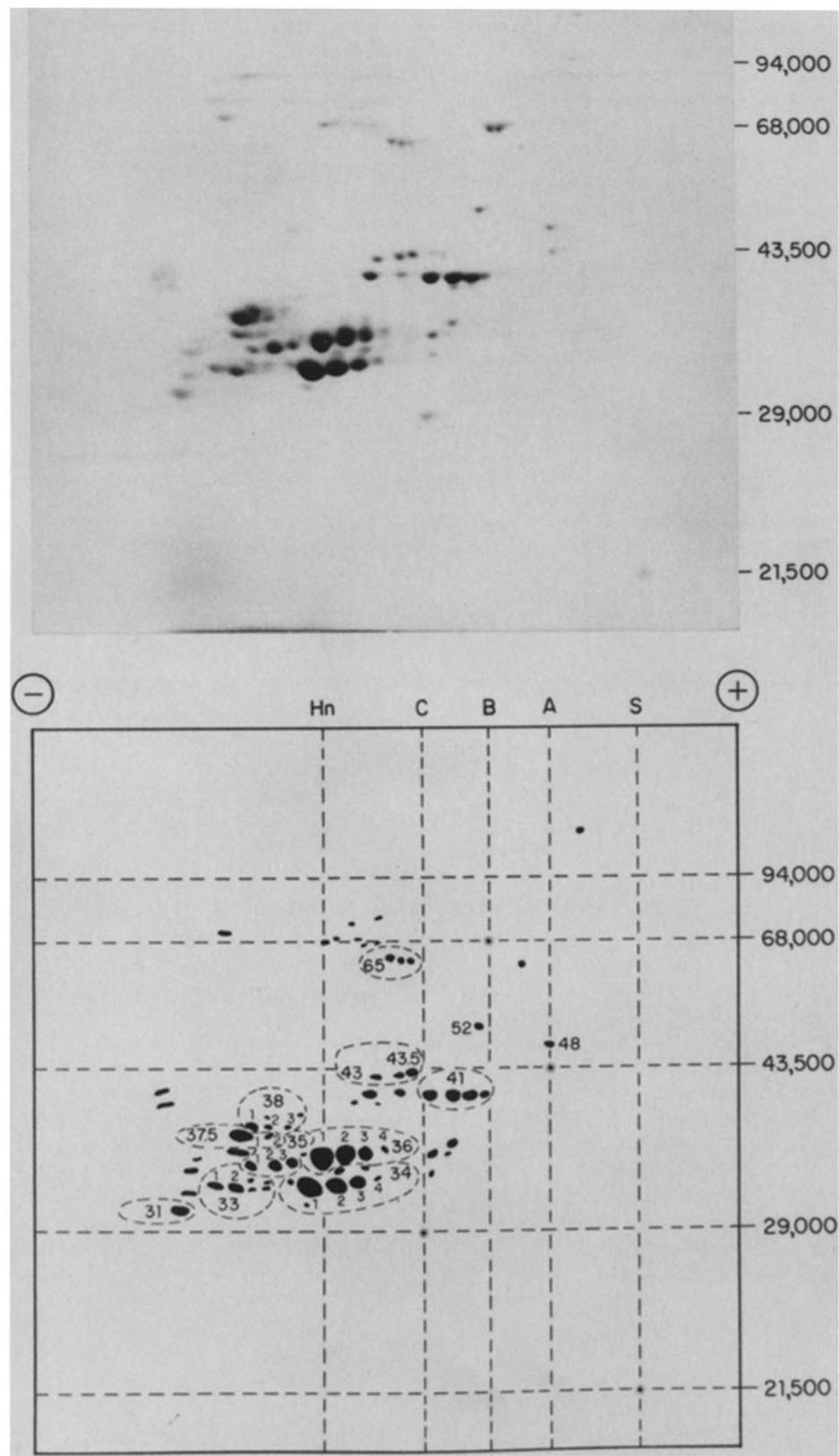


FIGURE 9 NEPHGE of rat liver hnRNP particles. 1,000 µg of sample was electrophoresed by the NEPHGE system as described in Materials and Methods. For description of *hn* standard, see Fig. 6.

The results presented here reiterate and extend earlier studies from this (30, 31, 35) and other laboratories (16, 84, 86) concerning the nature of the so-called nonhistone chromosomal proteins indicating that the concept of chromatin as a discrete entity of neatly packaged nucleic acid and protein is misleading and fails to truly account for the dynamic state of the cell.

A comparison of proteins of rat liver cytoplasm with SE and Tris washes of nuclei, and the final chromatin pellet by two-dimensional gel electrophoresis has resolved five "classes" of proteins: (a) distinctly cytoplasmic proteins; (b) ubiquitous proteins found in cytoplasm, nuclear washes and chromatin; (c) proteins found only in the nuclear washes; (d) proteins found in the nuclear washes and final chromatin pellet; and (e) proteins found only in the final chromatin fraction.

Cytoplasmic Proteins

Cytoplasmic proteins are present in the early (SE) nuclear washes and absent from the Tris washes and final chromatin. By electron microscopy these nuclei appear very clean with few cytoplasmic tags (not shown). However, whether or not the cytoplasmic contaminants are loosely bound in the nucleoplasm or associated with the outer nuclear membrane is not clear at this time. Others have investigated the extent of cytoplasmic contamination of chromatin preparations by various

methods, and in most cases the values are < 6% (16, 86, 98, 102). Most of these studies involve adding labeled cytoplasmic proteins to nuclei during processing. This may not reflect the real extent of cytoplasmic proteins in the nucleus as cytoplasmic "binding" sites could already be saturated. On the other hand, our results suggest that some cytoplasmic proteins thus labeled may, in fact, not be contaminants at all but "nuclear" proteins of potential regulatory, transport, structural, or enzymatic function distributed throughout the cell. The most satisfactory way of approaching this problem is by means of enucleation-fusion studies of the type reviewed by Bonner (19).

Ubiquitous Proteins

The second class contains the ubiquitous proteins (single-hatched in the appropriate figures). As discussed in the Results, we believe that the 43,000-dalton protein is actin. Actin is present in virtually all cells and has often been reported present in the nucleus, especially in organisms with an intranuclear spindle apparatus (24, 30, 57, 69, 89). Although our protein does not precisely co-migrate with rat skeletal muscle actin (standard), it is known that actins of muscle and nonmuscle cells are different in charge, with nonmuscle actin being slightly more basic (47, 97).

The amount of actin reported to be present in nuclei from a

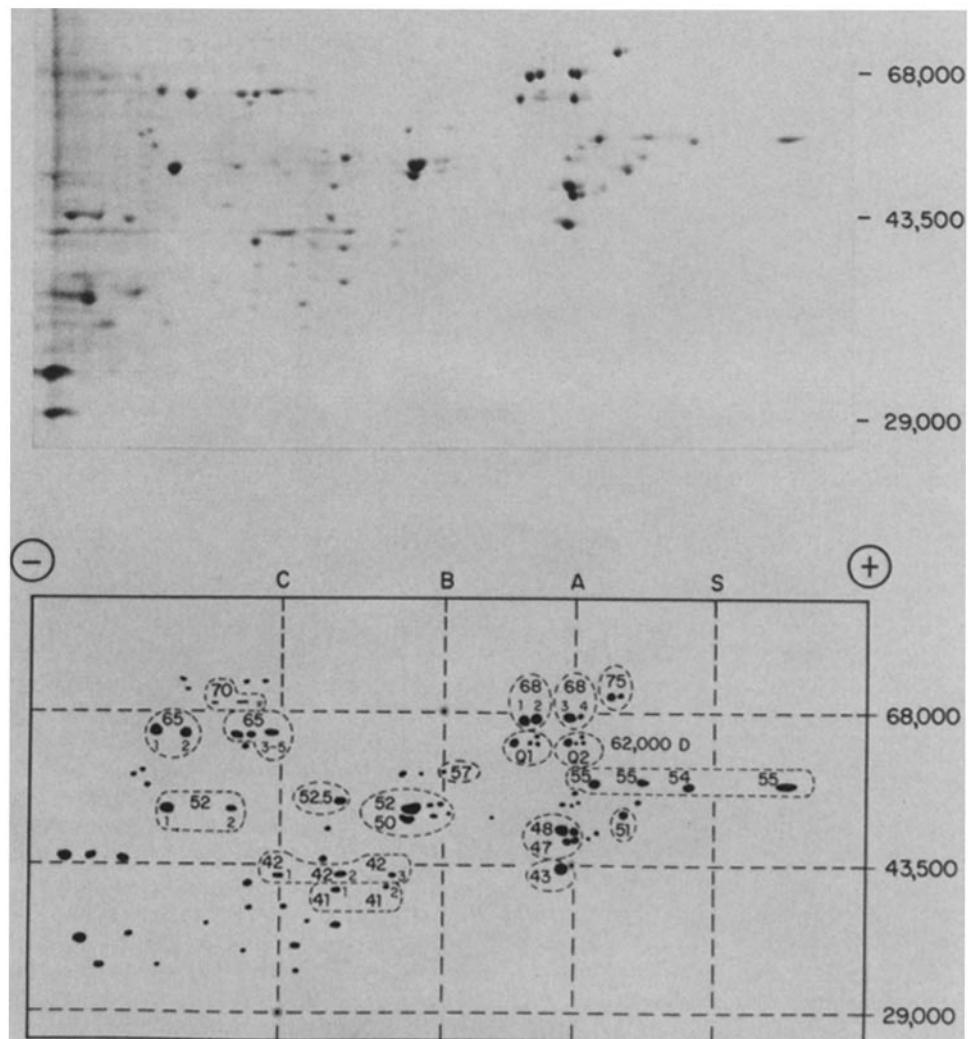


FIGURE 10 Standard two-dimensional gel electrophoresis of rat liver chromatin proteins. Chromatin was prepared by the method of Bonner et al. (18), and 1,200 µg was run as described in Materials and Methods.

variety of organisms varies considerably and has been the subject of some controversy (68). Specifically, with regard to rat liver, Douvas et al. (39) reported actin to be a major chromosomal protein. It, along with myosin, a breakdown product of myosin, α - and β -tropomyosin and tubulin, consti-

tuted 38% of the NHP of rat liver chromatin isolated by washing chromatin prepared from whole liver homogenates. Quantitation of these proteins in our two-dimensional gels (Table II) indicates that actin constitutes ~1.7% of the total rat liver NHP. We do not see α - or β -tropomyosin. Although we

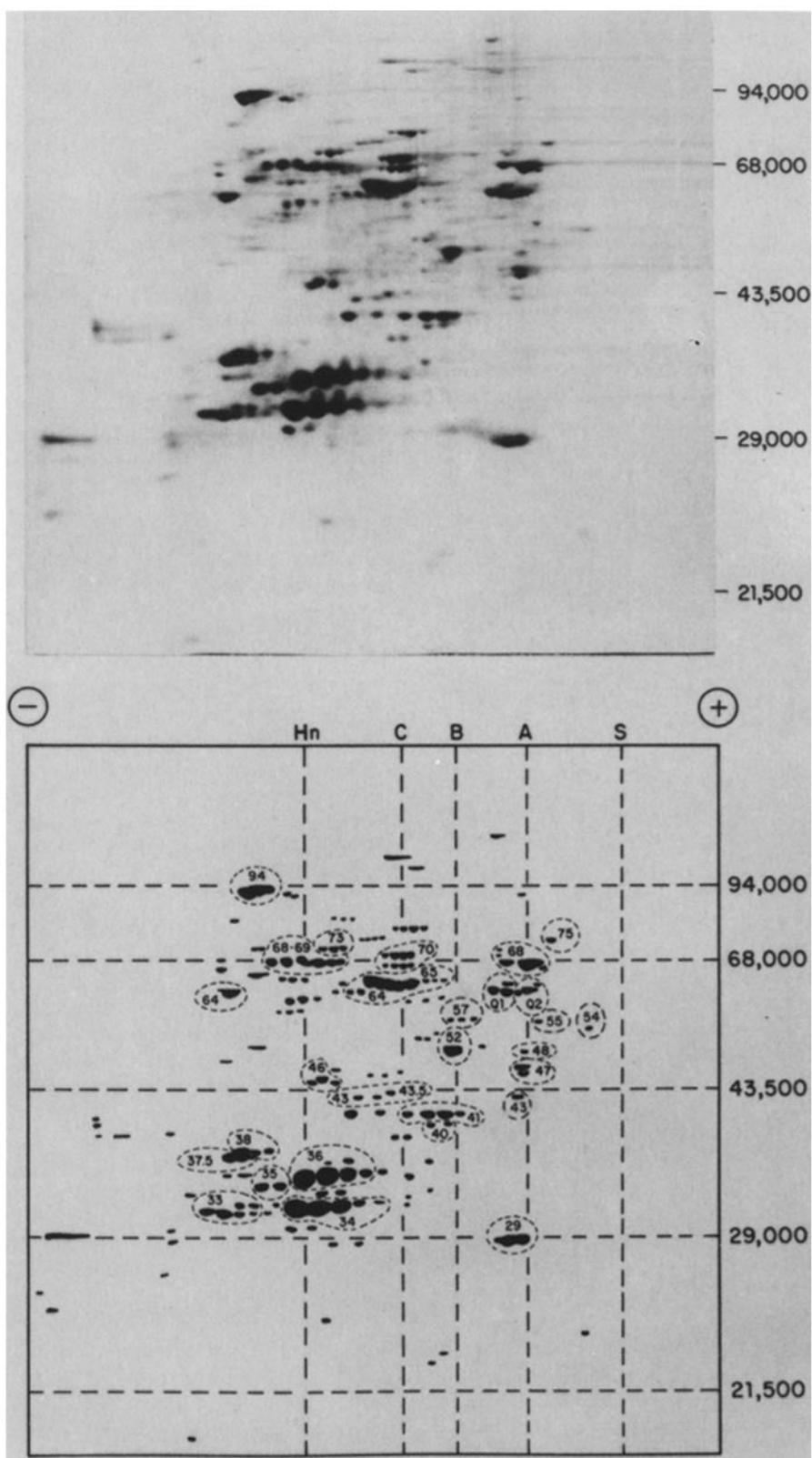


FIGURE 11. NEPHGE of rat liver chromatin proteins. 1,500 μ g of chromatin prepared by the method of Bonner et al. (18) was run in the NEPHGE system as described in Materials and Methods.

do not observe myosin, this protein is difficult to visualize in two-dimensional gels because it produces a diffuse, feathery pattern. The 100,000-g cytosol supernate (Fig. 2) and the SE washes (Fig. 4) show a faint doublet (53.5 protein of cytoplasm, Fig. 2) that co-migrates with purified rat brain tubulin. This, however, is missing from the unwashed nuclei and chromatin (Fig. 3, 10, and 11). Therefore, we conclude that contractile proteins probably constitute <5% of the NHP of rat liver. Comings and Harris (31) discussed previously the potential for excessive contamination of chromatin when it is isolated from whole cell lysates rather than from isolated nuclei.

The ubiquitous 75,000-dalton polypeptide is also present in gels of nuclear matrix and nuclear membrane preparations (Fig. 3 and 8). This may be the protein associated with poly A tails of the mRNA of polyribosomal complexes (8, 17, 61, 91).

Two of the four separate 68,000-dalton polypeptides are in the ubiquitous class. The grouping just slightly more basic than actin, which is represented by a charge doublet in most gels, is prominent in the nuclear matrix and is seen as a minor component of nuclear membrane preparations. This is consistent with the findings of Krohne et al. (64) who observed a protein of ~66,000 mol wt loosely attached to rat liver nuclear pore complexes, and with an earlier observation by Comings and Harris (31) that presumptive nuclear matrix proteins of this molecular weight can also be found in the cytoplasm. The other major, more basic, 68,000-dalton group (*C-B*) is markedly diminished in the Tris washes and, although it is not easily observed in the gels presented here of chromatin in Fig. 10 and 11, we have observed it in numerous other chromatin preparations.

The identity of the 57,000-dalton protein of the same isoelectric point as BSA may be the same protein reported by Barrett and Gould (7) to be the only protein they found common to the nonhistone fractions of rat liver, chicken liver, and chicken reticulocytes. They compared the nonhistones of these tissues using two-dimensional gel electrophoresis and characterized this protein as having a molecular weight of 56,000 daltons and a pI of 6.6, both values being essentially identical to those we observe.

Nuclear Wash Proteins

The third class of proteins (Fig. 4 and 5, double-hatched) is represented by the acidic species at 94,000, 25,000, 22,500, and 20,500 mol wt. These are present in all washes but absent from the final chromatin pellet. Thus, they are not totally removed by the SE washes but appear to be by Tris washes. The 25,000-dalton protein is most prominent in the Tris washes. It is not HMG-1 (50) because it lacks charge heterogeneity (52) and behaves differently in two-dimensional gel electrophoresis from purified HMG proteins of calf thymus and mouse liver (Conner and Comings, manuscript in preparation). That these proteins (particularly the 25,000-mol wt species) become more prominent in the Tris washes could reflect their increased solubilization as a result of the nuclear swelling properties of the hypotonic Tris solution, thereby allowing easier access of the solvent to these proteins on the chromatin. A similar situation may hold for the characteristic group of proteins in the 31,000–33,000 range (pI near actin) which appear prominently only in the Tris washes (Figs. 5 and 6). The behavior of these two sets of proteins (31,000–33,000 group vs. the proteins at 20,500, 22,500, 25,000, and 94,000 daltons) may reflect differing degrees of accessibility in the chromatin by the wash buffers.

Nuclear Wash and Chromatin Proteins

The fourth class of proteins were those present in all nuclear washes and final chromatin pellet. These are represented in the gels by the acidic group of molecular weight 54,000–55,000 (double-hatched, Fig. 3–5), some of which are components of the nuclear matrix. Additional matrix proteins which fall into this category include Q2 and the species at 48,000 Å. Although it is not obvious from Fig. 11 that the double-hatched grouping at 54,000–55,000 daltons is present in its entirety in the final chromatin pellet, many gels run of chromatin in the standard system (Fig. 10) show them to be consistently present.

Chromatin-specific Proteins

A 68,000-dalton protein seen on some standard gels of chromatin preparations (not shown) is one of the most acidic proteins we have observed in any gel (pI in urea of <5) and represents the fifth class of protein seen in our gels. This protein and the major nuclear membrane-matrix protein at 68,000 Å are the only major proteins we observe which are not obviously soluble in the wash buffers but which appear in the final washed pellet. They, along with certain minor species we observe, must represent very insoluble NHP.

Nuclear Matrix Proteins

By one-dimensional gel electrophoresis, the major proteins of the nuclear matrix have been observed as a set of three in the 60,000- to 75,000-dalton range (9, 11, 13, 32). Two-dimensional gel electrophoresis (Fig. 7) indicates that these are composed of three distinct subsets, an acidic set consisting of four isomers of polypeptide 68 and Q1 and Q2 with isoelectric points similar to actin, an intermediate set consisting of proteins 70 and 65 with isoelectric points near carbonic anhydrase, and a basic set (69 and 73) consisting of proteins with isoelectric points around the major hnRNP 34,000-dalton species. One acidic nuclear matrix-associated protein, protein 43, apparently is actin. Its presence in the well-washed matrix preparations suggests that it may be firmly bound to the matrix. Although it could play a role in the contractility of the matrix, Wunderlich and Herlan concluded that, because matrix contractility was not dependent on ATP, an actin-myosin system was not involved (100).

Approximately one-third of the total nuclear matrix proteins are composed of hnRNA proteins when the matrix is isolated in the presence of protease inhibitors. There are at least two possible interpretations of this: (a) there is an intimate association between the nuclear matrix and at least part of the hnRNP. This is consistent with the suggestions of Pogo and co-workers (43, 71, 76) and of Herman et al. (55); or (b) The hnRNP are simply not completely washed out of the nuclei during the preparation of the nuclear matrix. Because of the extensive washing of the nuclear matrix and its treatment with RNase followed by further washing, and the presence of large amounts of hnRNA proteins despite electron microscopy evidence of the disruption of the nuclear membrane in the majority of the nuclei, we believe that the first interpretation is more likely. The reported presence in lampbrush chromosomes of hnRNP particles that appear completely free of any attachment to nuclear matrix (75) doesn't rule out such an association. There may be different mechanisms for different rates of transcription, only some of the hnRNP may be involved in such matrix complexes and/or the situation with lampbrush

chromosomes in diplotene may not be typical of interphase nuclei.

Although both of the two major subunit proteins of hnRNP are believed to be involved in formation of the 30S–40S particles, they are not equally present in the nuclear matrix. Protein 36 was much more prominent than protein 34 (Fig. 7). One possibility for this is that some hnRNP may be preferentially complexed to matrix through an interaction with protein 36, although an equally tenable, though potentially less exciting, explanation could be the selective removal of protein 34 by the high salt and nuclease treatment in the preparation of matrix.

This apparent association between the nuclear matrix and the hnRNP implies that the matrix may play a role in hnRNA processing and translocation to the cytoplasm (43, 76). The association of nascent DNA with the matrix (10, 12) suggests that it also plays a role in DNA replication. Because they are at the heart of the action of two such vital processes, one wonders whether the nuclear matrix proteins are more than structural. Do they play an active role in these functions? Do they have some enzymatic activity? Oncogenic viruses are intimately involved with the nuclear matrix (9, 56), and some carcinogens show a preferential association with the matrix. What role does the nuclear matrix play in the altered metabolism of cancer cells? Berezney (9) has recently reviewed some of these questions.

Proteins of hnRNA

Many studies on the proteins of hnRNA using one-dimensional SDS gel electrophoresis have been reported. Most workers report two major polypeptides from 32,000 to 38,000 daltons with variable numbers of minor polypeptides (15, 53, 58, 74, 77, 93). Very few workers have successfully used two-dimensional gels. This is caused in part by the very basic nature of these proteins that some have termed the "histones of RNA," implying an analogy between the hnRNP particles and the nucleosomes (14, 85). Karn and co-workers (58) side-stepped the problem by using acid-urea gel electrophoresis in the first dimension and SDS gel electrophoresis in the second. Five major polypeptides and seven minor polypeptides at molecular weights of 29,000–42,000 daltons constituted 75% of the hnRNP. 13 minor proteins of higher molecular weight were present. Pagoulatos and Yaniv (83) used the standard O'Farrell procedure (78) with a pH gradient of 5–8 and visualized only the acidic hnRNP. Beyer et al. (15), also using the standard two-dimensional gel procedure, were occasionally able to visualize some of the more basic proteins from mammalian cells.

As shown in Fig. 9, using the Samarina technique (88) for isolation of 30–40S particles, we find two major sets of proteins, the 34 set composed of four charge isomers of decreasing size with increasing acidity, and the 36 set, slightly shifted in the acidic direction, but otherwise similar, with four charge isomers. These and other proteins that we find are highly reproducible (cf., Figs. 6, 9, and 11). Not counting the charge isomers, there were 12 distinct hnRNA proteins (Table II).

The only other study of rat liver hnRNPs using NEPHGE showed patterns very similar to ours (94). With rat liver 40S particles isolated by the procedure of Samarina et al. (88) or hnRNP isolated by the procedure of Pederson (84), Suria and Liew also found that the major hnRNPs consist of two sets between 32,000 and 42,000 daltons, each showing multiple-charge isomers. These proteins constituted 70% of their hnRNP.

There were 22 minor proteins, the most prominent being a pair of 62,000-dalton proteins in the hnRNP preparation. The patterns for the 40S particles isolated in matrizzimide gradients by the method of Karn et al. (58) were essentially the same. Suria and Liew also observed that the hnRNPs formed a significant part of the total chromatin proteins isolated in phenol.

Another more recent study, by Brunel and Lelay (21), also using the NEPHGE system of O'Farrell et al. (79), examined hnRNP particles of HeLa, mouse L and Chinese hamster ovary cells. They found many similarities in patterns among the three species, particularly in the major basic group clusters between 25,000 and 40,000 mol wt, which generally correspond to our 34,000–36,000-dalton group. An important finding was that the overall pattern of major polypeptides observed was not appreciably different between the 30–40S particles and larger particles of a more heterodisperse nature. A similar observation has been made by Suria and Liew (94). However, it was reported that a greater number of phosphorylated species appear with increasing particle size (21).

A more specific comparison between our work and that of Brunel and Lelay is again difficult because of the differences in data presentation. Although the overall patterns were somewhat different than either ours or those of Liew's group (although several similarities do exist), these variances may reflect differences in technique and tissues studied. Attempted comparisons of this type reiterate the need for more standardization and methods of precise comparison among different laboratories.

Nuclear Membrane-Lamina Complex

Electron microscopy of our nuclear membrane preparation shows linear structures representing the inner nuclear membrane associated with a more diffuse, feathery component (34). The presence of such proteinaceous filaments extending from the undersurface of the nuclear membrane to intermix with the outer portions of the peripheral heterochromatin was suggested by immunological studies of Gerace et al. (49) and Krohne et al. (63). The latter authors were concerned that they could not rule out the possibility of selective stabilization or protection of proteins in the periphery to explain the peripheral location of the proteins. The present study, using different techniques, comes to the same conclusion that the major portion of the nuclear membrane proteins (68, isomers 4 and 5 in Fig. 8) is a distinct subset of the nuclear matrix proteins that are found only in the nuclear membrane.

Nucleolar Matrix Proteins

NEPHGE of the nucleolar matrix (not shown) indicates that these proteins are distinct from the intranuclear matrix and nuclear membrane-lamina proteins. These are discussed in more detail elsewhere (34).

NHP of Chromatin

As mentioned previously, a standard method of preparing chromatin is to wash isolated nuclei twice with buffer solution of saline plus a chelating agent to remove nuclear sap proteins, then to wash the nuclei several times with hypotonic Tris buffers to swell the chromatin (18). In some preparations, chromatin is also sheared and any particulate matter is removed by centrifugation. The prevailing assumption through many years has been that the NHP remaining on this chromatin are

primarily involved in gene regulation. However, previous studies of nuclear proteins in our laboratory (26, 30, 32) and others (84, 86), using one-dimensional SDS gel electrophoresis indicated that the vast majority of electrophoretically visible NHP are derived from the matrix or hnRNP. The present two-dimensional gel electrophoresis studies confirm this conclusion. Approximately 80% of the mass (not number) of NHP of whole unwashed nuclei is derived from the intranuclear matrix, nuclear membrane, nucleolar matrix, and hnRNP. Furthermore, when the proteins of the well-washed nuclei and chromatin were analyzed by standard two-dimensional gel electrophoresis and NEPHGE, they were found to be essentially very similar to those of unwashed nuclei (Figs. 3, 10, and 11, and unpublished results).

Our finding that the hnRNPs are especially prominent in the chromatin preparations we've employed (18) substantiates reports by many investigators that the hnRNA may remain tightly associated with the chromatin (6, 16, 60, 84, 95). The NEPHGE studies of 11S monomer particles of chromatin also show amounts of hnRNP comparable to that of whole chromatin (70). Although disruption of nuclei by sonication as an alternative method of chromatin preparation has been reported to result in a lower percentage of hnRNP proteins associated with chromatin (16), this may be caused by the shearing of the hnRNA-protein complex from the chromatin as a result of sonication.

Although other groups of researchers have studied the NHP by two-dimensional gel electrophoresis (7, 73, 101), the type of systems used or the methods of presenting data generally do not allow convenient or accurate comparisons with our results. Similarities between our studies and that of Liew's group (70, 94) were discussed above.

Peterson and McConkey (86) reported a comparative study of cytoplasmic, nucleoplasmic, and chromatin proteins of HeLa cells employing the standard O'Farrell urea-NP-40 system using ³⁵S-methionine-labeled samples. This allowed detection of minor NHP, and the number of components they observed in chromatin was ~500 compared to the 100–200 we can detect with Coomassie staining. It is again impossible to make direct comparisons between specific proteins of the two studies, but their observation that several dozen abundant proteins are common to cytoplasm, nucleoplasm, and chromatin is consistent with our findings, given the different sensitivities of the two methods (labeled vs. unlabeled proteins).

Final Comments

In comparison to one-dimensional gel electrophoresis, especially of nuclear matrix, our two-dimensional gels show a relative paucity of high molecular-weight species. This is unlikely artifactual since we have clearly observed proteins of 250,000 daltons and greater in some preparations (29). Also, heavily loaded gels of nuclear proteins reveal several species between 90,000 and 200,000 daltons. Protein bands in one-dimensional SDS gels may be composed of multiple minor species of different isoelectric points which are individually too small to be seen in Coomassie blue-stained, two-dimensional gels. We have observed this in nucleolar matrix preparations (not shown).

We have stated earlier that the term "chromatin" must be considered valid only as an operational definition and we wish to emphasize here that, on the basis of our results, other nuclear components such as matrix and hnRNP particles should probably be viewed in the same manner. Furthermore, although we

cannot exclude the possibility of some contamination of certain nuclear fractions with components of others, we believe that given the remarkable reproducibility of our gels from preparation to preparation, in terms of both the specific polypeptides observed and the relative amounts of each, our results truly represent the associations of nuclear components suggested in this paper. This implies that perhaps less emphasis should be placed on the "compartmentalization" of nuclear substructures and more on the nucleus as a dynamic, complex interaction of functionally (and structurally) related components which together carry out the dictates of an organism's genetic information.

We would like to identify as many of the nuclear proteins as possible and to encourage correspondence from other groups of investigators willing to submit rat liver nuclear enzymes and other purified NHP for electrophoresis.

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