Nuclear Nonhistone Proteins in Murine Melanoma Cells

I. Quantitative Isolation and Fractionation

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Nuclear nonhistone proteins (NHP's) have been implicated as regulatory agents involved in controlling genetic expression. Utilizing murine melanoma cells, we describe a method for isolating and fractionating NHP's which greatly increases the yield of these proteins as well as the level of resolution required for detecting small differences in particular NHP's. Mouse melanoma cells were grown in medium labeled with [3H]leucine. Following 48 hr of incubation, the cells were harvested and nuclei isolated. The NHP's were extracted from the nuclei in a series of steps which yielded four major fractions: NHP₁, NHP₂, NHP₃, NHP₄. This method solubilized 80-90% of the protein from the nuclear homogenate. The NHP fractions were then separated on DEAE-cellulose columns in a series of salt steps increasing in concentration from 0.05 to 0.50 M NaCl, followed by steps of 2 M NaCl and 4 and 7 M guanidine-hydrochloride. The 40 NHP fractions eluted from these columns were further separated on polyacrylamide-SDS gels and ranged in molecular weight from 9000 to 110,000 daltons. Differences were observed in the electrophoretic pattern of each of these 40 fractions. The high resolution of these fractionation procedures greatly enhances the possibility of observing small changes in proteins which may play a role in gene regulation.

INTRODUCTION

Nonhistone proteins are involved in the regulation of gene expression in certain prokaryotic systems (1-4). Nuclear nonhistone proteins (NHP's) associated with chromosomes may play an analogous role in eukaryotic cells (5-11). They satisfy many of the criteria for a regulatory protein: they are heterogenous (8, 10, 12-16); they possess both tissue and species specificity (7, 8, 13, 17); they exhibit a high affinity for DNA (7, 8, 18); and they influence DNA template activity (6, 8, 19-24). No method, however, has yet been described in eukaryotes which quantitatively recovers these proteins under conditions which permit resolution sufficiently sensitive to detect changes in specific proteins which may be involved in gene-regulation.

We describe here a method for extracting NHP's from melanoma cells under conditions which have been chosen to minimize denaturation and loss of potential biological activity, i.e., binding to DNA and template activity, maximize NHP yield, and greatly increase resolution of these proteins.

MATERIALS AND METHODS

Culture Methods

Mouse melanoma cells (Cloudman S91 NCTC 3960 (CCL 53)) were obtained from the American Type Culture Collection Cell Repository. Monolayer cultures were grown in 250-ml Falcon culture flasks, 75-cm² growth area, at 37°C, in Ham's nutrient mix F-10 (GIBCO) supplemented with 2% fetal calf serum (GIBCO), 10% horse serum (GIBCO), 100 units/ml of penicillin, 100µg/ml of streptomycin, and 1.2 mg/ml of sodium bicarbonate.
Each flask was seeded with $1 \times 10^6$ cells. After 48 hr fresh F-10 medium which lacked thymidine and leucine was added to the flasks. To this medium was added 8.1 $\mu$Ci/ml of L-[4, 5-3H]leucine (New England Nuclear), 0.7 $\mu$g/ml of thymidine, and 1.3 $\mu$g/ml of L-leucine to give a final concentration of leucine of 2 $\mu$g/ml (15% of leucine normally present in Ham's F-10 mixture). The concentration of thymidine was the same as that normally present in Ham's F-10 mixture. Cells were grown in this medium for 48 hr.

**Isolation of Nuclei**

Cells were released from the flasks with Ca$^{2+}$, Mg$^{2+}$-free Tyrode's solution containing 5 mM EDTA, and sedimented for 5 min at 500g. The pellets were sedimented twice in 0.14 M NaCl and were resuspended in a Potter–Elvehjem Teflon-pestle glass homogenizer at a concentration of $4 \times 10^8$ cells/60 ml of H buffer: 0.05 M tricine, pH 7.5; 0.02 M KCl; 0.005 M MgCl$_2$; and 0.35 M sucrose to which was added 3 mg/ml of L-leucine. The cells were homogenized at 1000 rpm with a Teflon pestle at 4°C and centrifuged at 5000g for 10 min. This homogenization procedure was repeated twice. The pellets were gently homogenized in 60 ml of a solution of 2.2 M sucrose, 1.5 mM CaCl$_2$ (25) in a Dounce homogenizer using both A and B balls. The suspension was centrifuged in an SW 50.1 Spinco rotor for 30 min at 30,000g. The resulting pellet contained the purified nuclei. The integrity and purity of the nuclei were assured by light and electron microscopy.

**Extraction of Nuclear Nonhistone Proteins**

Extractions of the NHP's at 4°C were based on the methods of Spelsberg et al. (26) as outlined in Fig. 1. The nuclear pellets were homogenized with a Teflon-pestle glass homogenizer in NHP$_1$ buffer containing 10 mM PO$_4$, pH 6; 2 M NaCl; 5 mM urea; 1 mM MgCl$_2$; and 0.1% β-mercaptoethanol at a concentration of 0.4 mg of protein/ml and were centrifuged in a Spinco 40 rotor for 24 hr at 105,000g. The supernatant fraction contained the first nonhistone protein fraction (NHP$_1$) plus the histones. The pellet, which included the remaining nonhistone protein plus DNA, was rinsed with distilled water, resuspended in NHP$_2$ buffer, 2 mM Tris–HCl, pH 7.5 (0.6 mg/ml) with a Teflon-pestle glass homogenizer and dialyzed for 2 hr against the same solution. This suspension was then centrifuged at 800g for 10 min to sediment the insoluble protein, NHP$_2$. The supernatant fraction, containing DNA with the remaining nonhistone proteins, was further separated by addition of 2 vol of NHP$_3$ buffer containing 15 mM Tris, pH 8.5; 3.0 M NaCl; 7.5 mM urea; 1.5 mM NaHSO$_4$; 1.5 mM EDTA; and 0.1% β-mercaptoethanol. This suspension was vigorously mixed and allowed to stand at -20°C for 2 to 3 hr. It was then centrifuged for 36 hr at 105,000g to sediment the DNA bound to the remaining protein fraction (NHP$_4$). NHP$_3$ remained in the supernatant fraction. Separation of NHP$_4$ from DNA was based on a method of Teng et al. (8). The pellet containing DNA and NHP$_4$ was suspended in 5 vol of 0.1 M Tris–HCl, pH 8.4; 0.01 M EDTA; and 0.14 M β-mercaptoethanol (Buffer A). This suspension was gently mixed with an equal volume of cold phenol saturated with Buffer A and allowed to stand for 18 hr at 4°C. The mixture was homogenized briefly with a Teflon–glass homogenizer and centrifuged at 12,000g for 10 min. The phenol phase was collected and dialyzed against 100 vol of 0.1 M acetic acid containing 0.14 M β-mercaptoethanol for 3 hr at 4°C. The dialysis was continued against fresh 0.1 M acetic acid, 0.14 M β-mercaptoethanol until the phenol phase was reduced to about one-third of its original volume. The phenol phase was removed and dialyzed against 0.05 M acetic acid, 9.0 M urea, 0.14 M β-mercaptoethanol for 22 to 24 hr at 13°C.
FIG. 1. Outline of procedures used for extraction and fractionation of nuclear nonhistone proteins from murine melanoma cells (see Materials and Methods).

Dialysis was continued for 2 hr in NHP$_4$ buffer containing 0.1 M Tris–HCl, pH 8.4; 8.6 M urea; 0.01 M EDTA; and 0.14 M β-mercaptoethanol. NHP$_4$ was then frozen at −70°C.

**Column Chromatography**

All of the following procedures were carried out at 4°C. NHP$_1$ was separated from the histones by column chromatography on Bio-Rex 70 (200–400 mesh, Na form, Bio-Rad Laboratories) according to the procedure of Levy et al. (27). The de-fined resin was packed by gravity to a final height of 11 cm in a siliconized glass column, with an inside diameter of 9 mm. The Bio-Rex column was equilibrated with the running buffer which contained 0.1 M NaPO$_4$, pH 7.0; 6.0 M urea; 0.35 M guanidine hydrochloride (G–HCL); 0.1% β-mercaptoethanol. NHP$_1$ was dialyzed for 20 hr against 100 vol of the running buffer. The nonadsorbed peak contained the NHP’s. The histones, which were retained, were then eluted with 4 M G–HCL in the running buffer, followed by a step of 7 M G–HCL. The histones were frozen at −70°C.

NHP$_1$, NHP$_2$, and NHP$_3$ were each fractionated on DEAE-cellulose (DE-52, Whatman). The de-fined resin was packed by gravity in a siliconized glass column, 9 mm x 11 cm, and equilibrated for at least 20 hr in 0.01 M Tris, pH 8.5; 3.0 M urea, 0.1% β-mercaptoethanol. NHP$_1$, NHP$_2$, and NHP$_3$ were each dialyzed for at least 20 hr in 50 to 100 vol of the above buffer, with one change. NHP$_3$ was briefly homogenized in DEAE buffer (0.10 mg of protein/ml of buffer) before being dialyzed. The samples were applied to the columns and the initial nonadsorbed peaks collected. The bound NHP’s were eluted from the column with NaCl in a series of seven to eleven steps increasing in concentration from 0.05 to 0.5 M. Residual protein was recovered in steps of 2 M NaCl and 7 M G–HCL. The columns were monitored by counting aliquots of each fraction in 10 ml of Scintisol (Isolab, Inc., Akron, Ohio) on a Nuclear Chicago Mark II scintillation counter. The fractions were pooled and frozen at −70°C.
Gel Electrophoresis

The NHP and histone fractions were subfractionated on SDS–polyacrylamide gels according to the method of Teng et al. (8). Gels, 90 × 6 mm, containing 10% acrylamide, 0.2% N, N'-methylenebisacrylamide, 0.075% ammonium persulfate, and 0.075% TEMED (N,N,N',N'-tetramethylthlenediamine) in 0.1 M NaPO₄, pH 7.4, 0.1% SDS, were prerun for 30 min at 4.5 V/cm in the same buffer. The protein samples were dialyzed at 5°C for 4 to 24 hr against several changes of distilled water (at least 100 vol) containing 0.1% β-mercaptoethanol. The samples were then lyophylized and redissolved in 0.01 M NaPO₄, pH 7.4; 1% SDS and allowed to sit at room temperature for 3 hr. They were further dialyzed overnight against 0.001 M NaPO₄, pH 7.4; 0.1% SDS; and 0.14 M β-mercaptoethanol. After dialysis, 100 µl of the sample was mixed with 25 µl of 42% sucrose and 0.01% bromophenol blue in the dialysis buffer. The sample (100–200 µl), containing 10,000–100,000 cpm, was applied to the gel. Electrophoresis was carried out at room temperature at 4.5 V/cm for 6 to 7 hr. The gels were removed from the tubes and stained overnight in 0.2% brilliant Coomassie blue in methanol: acetic acid:water (5:1:4) and then destained in methanol:acetic acid:water (1:1:8). Alternatively, gels were removed from the tubes and frozen at −20°C. The frozen gels were then sliced transversely into 1-mm sections on a Mickle gel slicer and placed in scintillation vials containing 8 ml of the following mixture: 20 ml of 4 N NH₄OH; 100 ml of NCS solubilizer (Amersham/Searle Co.); and 1000 ml of Liquiflour–toluene (1:24) (New England Nuclear Corp.) (28). The vials were allowed to stand at room temperature for several days to elute the radioactivity from the gel. The samples were counted in a Nuclear Chicago Mark II Scintillation Counter. Counting efficiency was 43% for ³H.

The molecular weights of the various proteins were then estimated from the linear relationship between electrophoretic mobility and the logarithm of the molecular weights of the protein standards (29). These included: bovine albumin, fraction V, monomer, 68,000 daltons (Nutritional Biochemicals); human myeloma IgG, light chain, 22,000 daltons, and heavy chain, 53,000 daltons (gift of R. Rosenstein); bovine pancreatic deoxyribonuclease I, 31,000 daltons (Sigma); and horse heart cytochrome c, 12,400 daltons (Sigma).

Analysis

Protein was determined by the method of Lowry et al. (30) after precipitation with 5% trichloroacetic acid (4°C) and solubilization in 0.2 N NaOH (100°C). Bovine albumin was used as a standard. DNA was determined by the Burton diphenylamine procedure (31), using deoxyadenosine as a standard. DNA was extracted by precipitating the sample in 10% perchloric acid at 4°C and then solubilizing the nucleic acid in 10% perchloric acid at 70°C.

The various protein fractions were prepared for amino acid analysis by dialyzing them in 500–1000 vol of distilled water at 4°C with at least three changes. The samples were lyophilized and then hydrolyzed in evacuated tubes in 6 N HCl at 100°C for 24, 48, and 72 hr according to the method of Moore and Stein (32) and their amino acid compositions were determined on a Beckman 121M amino acid analyzer. Correction was made for hydrolytic loss for threonine, serine, valine, and isoleucine (32).

RESULTS

Isolation of Nuclear Nonhistone Proteins

The method of isolating nuclei avoids the use of detergents which might disrupt membranes and permit loss or denaturation of soluble nuclear proteins. The low-
speed centrifugation through 2.2 M sucrose also eliminated significant contamination of nuclei by cytoplasmic proteins. Electron microscopic examination of purified preparations by Dr. Gisela Moellmann showed nuclei with intact nuclear membranes and no visible cytoplasmic contaminants. The recovery of nuclei was determined by counting nuclei stained with methyl green before and after sucrose sedimentation and by DNA determinations using Burton's diphenylamine procedure (31). The yield of nuclei was approximately 75%.

The NHP's were isolated from the nuclei in a series of extractions yielding four fractions. This method solubilized 80–95% of the protein from the nuclear homogenate. The percentage of the nuclear protein recovered in each of these fractions was determined by the method of Lowry et al. (30) as well as by absorbance at 280 nm and was as follows: NHP1 plus histones, 60–75%; NHP2, 10–20%; NHP3, 3–5%; and NHP4, 0.3–0.4%. These values were reproducible from preparation to preparation. This procedure utilizes a series of buffers of alternating ionic strength which increase in pH from 6 to 8.5. Four NHP fractions are sequentially removed from DNA in higher yield than possible in a single-step separation. We have tried several such one-step separations of NHP's from DNA (27, 33) and our yields of NHP's ranged from only 30 to 38% of the total nuclear protein. Moreover, using our separation technique, the solubilized protein fractions (NHP1–NHP4) can then be dialyzed, with little loss, into buffers of sufficiently low ionic strength to permit binding to ion exchange resins for further fractionation.

NHP1 was separated from the histones on Bio-Rex, a cation exchanger. The nonadsorbed fraction contained the nonhistone protein (30–40% of total nuclear protein) while the adsorbed fraction contained the histones (30–35% of the nuclear protein). The total recovery from this procedure was 90–95%.

In order to confirm the separation of NHP's from histones on Bio-Rex, cells were labeled for 48 hr with [3H]-tryptophan (2.1 Ci/mmol) (New England Nuclear, 5 μCi/ml of medium). The cells were harvested and the NHP's isolated. NHP1 plus histones were separated on a Bio-Rex column. Since histones contain no tryptophan, all of the label should be associated with the nonhistone proteins (34). Ninety-five percent of the counts were recovered from the Bio-Rex separation, of which less than 6% was eluted with the histones. This indicated little contamination of the histone fraction by NHP's. NHP2 and NHP3, when chromatographed on Bio-Rex, were shown to contain negligible histone or other strongly basic protein. Stained SDS–polyacrylamide gels of each of the four NHP fractions are shown in Fig. 2. This figure demonstrates that each of the four fractions is highly heterodisperse.

Amino acid analysis of the various NHP fractions shows that their acidic amino acid content is greater than that of their basic amino acids as compared to the histones (Table 1). These ratios are similar to those found in mouse (14) and rat (8, 35–38) liver chromatin, pig cerebellar and pituitary chromatin (39), and human fibroblast nuclear proteins (40).

**Comparison of Nuclear Nonhistone Proteins**

NHP1, NHP2, and NHP3 were dialyzed into a low salt buffer and applied to DEAE-cellulose columns. Figure 3 shows the elution of NHP3 from the column with steps of increasing salt concentration. Approximately 60–80% of the added counts were recovered from the DEAE-cellulose columns. Elution profiles from linear salt gradients showed substantially less resolution that the step gradients.

Fractions eluted at each salt concentration from the DEAE-cellulose columns were pooled and further separated on SDS gels. The fractionation patterns showed
great diversity. NHP₁ was eluted in a series of 15 salt steps. The proteins ranged in molecular weight from 9000 to 110,000 daltons. Representative gel patterns of a number of these fractions are shown (Fig. 4). Proteins in the nonadsorbed material were mostly low molecular weight (Fig. 4b) in contrast to those eluted at varying salt concentrations which showed a wide range of molecular weights. There are differences in patterns of the peaks among the various fractions. The 0.075, 0.10, and 0.125 M NaCl (Fig. 4d) fractions differ from the 0.05 M fraction (Fig. 4c) in the loss of the 17,000 molecular weight proteins and the appearance of proteins of 36,000 daltons. There is a progressive decrease in the low molecular weight proteins (10,000–16,000) in the 0.125 and 0.150 M NaCl fractions (Figs. 4d and e) and the 35,000–40,000 molecular weight proteins in the 0.150 M fraction. As the NaCl concentration is increased to 0.175 M, 18,000 and 37,000 dalton proteins are eluted (Fig. 4f). The 0.20 and 0.250 M fractions are similar to this except for the loss of the 37,000 dalton proteins. As the salt concentration is increased to 0.30 M (Fig. 4g) and 0.40 M, a larger percentage of the protein is found in the 50,000–95,000 molecular weight range. The remaining NaCl fractions (0.5–2 M) show a progressive decrease in the high molecular weight proteins and only low molecular weight proteins (9000–17,000) are observed in the 2 M fractions (Fig. 4h). NHP’s displaying a wide

**FIG. 2.** Electrophoretic profile of NHP fractions on 10% SDS-polyacrylamide gels. Samples were prepared as described in Materials and Methods and electrophoresed following the protocol of Teng et al. (8). Direction of migration was from top (cathode) to bottom (anode) at 4.5 V/cm for 7 hr. Gels were stained in 0.2% brilliant Coomassie blue. (a) NHP₁, (b) NHP₂, (c) NHP₃, (d) NHP₄.
### TABLE 1
Amino Acid Analysis of Nuclear Nonhistone and Histone Proteins from Murine Melanoma Cells

| Amino acid      | Protein fraction* |          |          |          |          |
|-----------------|-------------------|----------|----------|----------|----------|
|                 | NHP<sub>1</sub>   | NHP<sub>2</sub> | NHP<sub>3</sub> | NHP<sub>4</sub> | Histones |
| Aspartic acid*  | 11.1              | 8.9      | 9.7      | 9.2      | 5.4      |
| Threonine       | 5.4               | 6.0      | 5.7      | 5.8      | 5.7      |
| Serine          | 6.9               | 9.5      | 8.6      | 8.4      | 8.0      |
| Glutamic acid*  | 12.2              | 11.9     | 11.6     | 11.1     | 7.6      |
| Proline         | 5.2               | 4.8      | 5.2      | 3.7      | 5.3      |
| Glycine         | 8.6               | 12.5     | 10.5     | 9.1      | 8.0      |
| Alanine         | 8.8               | 8.2      | 8.3      | 7.6      | 14.1     |
| Valine          | 6.2               | 6.4      | 7.5      | 6.5      | 6.8      |
| Methionine      | 0.9               | 0.7      | 0.4      | 1.7      | 0.4      |
| Isoleucine      | 4.1               | 4.5      | 3.6      | 5.1      | 3.4      |
| Leucine         | 8.6               | 8.2      | 7.9      | 10.6     | 6.0      |
| Tyrosine        | 2.5               | 2.7      | 2.3      | 3.0      | 2.5      |
| Phenylalanine   | 3.5               | 3.4      | 4.0      | 4.6      | 1.9      |
| Histidine**     | 2.3               | 2.1      | 3.7      | 2.4      | 1.7      |
| Lysine**        | 7.8               | 6.9      | 7.0      | 6.5      | 17.0     |
| Arginine**      | 6.9               | 4.5      | 4.7      | 4.8      | 7.0      |
| Acidic (A)*     | 23.3              | 20.7     | 21.3     | 20.3     | 13.0     |
| Basic (B)**     | 17.0              | 13.5     | 15.4     | 13.7     | 25.7     |
| A/B             | 1.37              | 1.53     | 1.39     | 1.48     | 0.51     |

* Values are expressed in moles/100 moles of recovered amino acids and are the average of three preparations except for NHP<sub>4</sub> which represents two analyses. Corrections were made for hydrolytic losses (see Materials and Methods).

Range of molecular weights (10,000–85,000 daltons) are again eluted at 4 M G-HCl (Fig. 4i) and 7 M G-HCl. Stained SDS gels of several of the NHP<sub>1</sub> fractions are seen in Fig. 5.

NHP<sub>2</sub> was eluted from the DEAE-cellulose column in a series of 11 steps of increasing salt concentration. Several of these fractions were combined before being separated by polyacrylamide-gel electrophoresis. The proteins ranged in molecular weight from 9000 to 90,000 daltons. Several of the gel patterns are shown in Fig. 6. In contrast to NHP<sub>1</sub>, only low molecular weight proteins (9000–15,000) were found in the 0.05–0.075 M NaCl fractions. However, as the salt concentration was increased, proteins of higher molecular weight were eluted. Proteins of 17,000–35,000 daltons appeared in the 0.1–0.15 M fraction and, along with these, 54,000–78,000 dalton pro-

![FIG. 3. Elution profile of NHP<sub>4</sub> on DEAE-cellulose. The sample and column were prepared as described in Materials and Methods. NHP<sub>4</sub> was eluted in a series of 11 salt steps increasing in concentration from 0.05 to 2.0 M NaCl followed by steps of 4 and 7 M G-HCl. Arrows (↓) indicate when each salt step was added to the column. One-milliliter fractions were collected.](image-url)
FIG. 4. Separation of NHP₁ fractions of SDS gels. The fractions were eluted from a DEAE-cellulose column in a series of salt steps and prepared as described in Materials and Methods. Samples were electrophoresed as described in Fig. 2. Gels were sliced transversely into 1-mm sections and each slice was counted for 20 min. (a) Molecular weight range of protein standards (see Materials and Methods), (b) flow through, (c) 0.05 m NaCl fraction, (d) 0.125 m NaCl fraction, (e) 0.150 m NaCl fraction, (f) 0.175 m NaCl fraction, (g) 0.30 m NaCl fraction, (h) 2.0 m NaCl fraction, (i) 4.0 m G–HCl fraction.

Proteins were eluted at 0.2–0.3 m NaCl (Fig. 6b). As the salt concentration was increased to 0.5 m NaCl (Fig. 6c) and 4 m (Fig. 6d) and 7 m G–HCl, there was a progressive increase in elution of higher molecular weight proteins (40,000–85,000 daltons).

NHP₃ was separated on a DEAE-cellulose column into 13 fractions. Several of the patterns of these fractions of gels are shown in Fig. 7. The proteins ranged in molecular weight from 8800 to 88,000 daltons. Like NHP₁, proteins in the flow through (Fig. 7b) and those eluting at 0.05 and 0.075 m NaCl were of low molecular
FIG. 5. Electrophoretic profiles of NHP, fractions on SDS gels. Fractions were eluted from a DEAE-cellulose column in a series of salt steps and applied to the gels as described in Fig. 2. Gels were run from top (cathode) to bottom (anode) at 4.5 V/cm for 7 h and stained in 0.2% brilliant Coomassie blue. (a) 0.3 m NaCl fraction, (b) 2.0 m NaCl fraction, (c) 4.0 m G-HCl fraction.

FIG. 6. Separation of NHP, fractions on SDS gels. The fractions were eluted from a DEAE-cellulose column and electrophoresed as described in Fig. 3. (a) Molecular weight standards, (b) pooled 0.2-0.3 m NaCl fractions, (c) 0.5 m NaCl fraction, (d) 4.0 m G-HCl fraction.
FIG. 7. Separation of NHP3 fractions on SDS gels. The fractions were eluted from a DEAE-cellulose column and electrophoresed as described in Fig. 3. (a) Molecular weight standards, (b) flow through, (c) 0.10 M NaCl fraction, (d) 0.125 M NaCl fraction, (e) 0.15 M NaCl fraction, (f) 0.20 M NaCl fraction, (g) 4.0 M G-HCl fraction.

weight (8000–16,000). Unlike NHP2, fractions of 0.10 M NaCl to 7 M G–HCl contained proteins with a wide range of molecular weights with some differences appearing between the various fractions. The 0.125 M NaCl fraction (Fig. 7d) differs from the 0.10 M fraction (Fig. 7c) in the increased amount of protein of 33,000 daltons. This increase is not seen in the 0.15 M fraction (Fig. 7e). The 0.20 M fraction has an 18,000 molecular weight peak (Fig. 7f) not seen in the preceding fractions. The 0.3 through 0.5 M NaCl fractions were similar to 0.20 M with only slight differences. Proteins eluted at 2 M NaCl and 4 M and 7 M G-HCl (Fig. 7g) ranged in molecular weight from 9000 to 50,000 (with no higher molecular weight peaks).

Since NHP4 contained less protein than the other fractions, the entire fraction was run on an SDS gel. Proteins in NHP4 ranged in molecular weight from 9000 to 90,000 daltons (Fig. 8).

DISCUSSION

In order to measure small changes among a potentially large number of NHP's it is essential to obtain the highest possible yield of these proteins, as well as to increase the level of resolution required for detecting small differences among them. We have modified a method (26) for isolating NHP's from mouse melanoma cells which optimizes their recovery and identification.
Many studies of NHP's have attempted to analyze unfractonated or less extensively fractionated preparations (8, 16, 23, 26, 38, 41–47). These methods are inadequate for detecting small changes in nonhistone protein, since the large number of minor protein species may be masked by the major proteins present (47). We have divided the NHP's into four fractions (NHP₁–NHP₄) (26) and have reduced the complexity of each fraction by means of extensive subfractionation on DEAE-cellulose columns followed by further separation on polyacrylamide gels. Gels from each of the 40 pooled fractions recovered from the columns were sliced into 80–100 sections, yielding a total of nearly 4000 subfractions. This method achieved a degree of resolution far greater than any previously described. As in any fractionation procedure involving step gradients, there appears to be some overlap of proteins with respect to molecular weight or electrophoretic mobility. We do not know if these are similar or different polypeptides. This determination would be accomplished by a functional analysis which we anticipate. However our initial aim was to separate these proteins into a large number of fractions in order to detect species present in small quantities.

In addition to providing a high-resolution technique for analyzing NHP's, our approach for isolating these proteins also differs from many previous efforts. A number of procedures for extracting NHP's is based on the assumption that chromatin is the optimal starting material (13, 14, 27, 35–39, 48–50). However, soluble nuclear proteins are discarded in these procedures. Our rationale was to increase our yield by extracting all of the nonhistone proteins from the nucleus. We do not know how many of the nuclear proteins which we have isolated play a role in genetic regulation, but it is possible to distinguish them from nonregulatory proteins by operational criteria such as ability to bind to homologous DNA as well as to regulate the rates and products of DNA transcription. These studies are currently in progress.

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REFERENCES

1. Jacob, F., and Monod, J., Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3, 318 (1961).
2. Ptashne, M., Specific binding the λ phage repressor to λ DNA. Nature (London) 214, 232–234, (1967).
3. Gilbert, W., and Müller-Hill, B., The lac operator of DNA. Proc. Nat. Acad. Sci. USA 58, 2415–2421 (1967).
4. Riggs, A. D., Bourgeois, S., Newby, R. F., and Cohn, M., DNA binding of the lac repressor. *J. Mol. Biol.* **34**, 365–368 (1968).
5. Paul, S., and Gilmour, R. S., Organ-specific restriction of transcription in mammalian chromatin. *J. Mol. Biol.* **34**, 305–316 (1968).
6. Teng, C.-S., and Hamilton, T. H., Role of chromatin in estrogen action in the uterus. II. Hormone-induced synthesis of nonhistone acidic proteins which restore histone-inhibited DNA-dependent RNA synthesis. *Proc. Nat. Acad. Sci. USA* **63**, 465–472 (1969).
7. Kleinsmith, L. J., Heidema, J., and Carroll, A., Specific binding of rat liver nuclear proteins to DNA. *Nature (London)* **226**, 1025–1026 (1970).
8. Teng, C.-S., Teng, C. T., and Allfrey, V. G., Studies of nuclear acidic proteins. *J. Biol. Chem.* **246**, 3597–3609 (1971).
9. O'Malley, B. W., Spelsberg, T. C., Schrader, W. T., Chytii, F., and Steggles, A. W., Mechanisms of interaction of a hormone-receptor complex with the genome of a eukaryotic target cell. *Nature (London)* **235**, 141–144 (1972).
10. Richter, K. H., and Serkeris, C. E., Isolation and partial purification of non-histone chromosomal proteins from rat liver, thymus, and kidney. *Arch. Biochem. Biophys.* **148**, 44–53 (1972).
11. Stein, G. S., and Baserga, R., Nuclear proteins and the cell cycle. *Adv. Cancer Res.* **15**, 287–330 (1972).
12. Elgin, S. C. R., and Bonner, J., Limited heterogeneity of the major nonhistone chromosomal proteins. *Biochemistry* **9**, 4440–4447 (1970).
13. Platz, R. D., Kish, V. M., and Kleinsmith, L. J., Tissue specificity of non-histone chromatin phosphoproteins. *FEBS Lett.* **12**, 38–40 (1970).
14. MacGillivray, A. J., Cameron, A., Krauze, R. J., Rickwood, D., and Paul, J., The non-histone proteins of chromatin, their isolation and composition in a number of tissues. *Biochim. Biophys. Acta* **227**, 384–402 (1972).
15. Le Stourgeon, W. M., Goodman, E. M., and Rusch, H. P., The nuclear acidic proteins from haploid and diploid cell states of *Physarum polycephalum*. *Biochim. Biophys. Acta* **317**, 524–528 (1973).
16. Seale, R. L., and Aronson, A. I., Chromatin-associated proteins of the developing sea urchin embryo. II. Acid-soluble proteins. *J. Mol. Biol.* **75**, 633–647 (1973).
17. Leob, J. E., and Creuzet, C., Electrophoretic comparison of acidic proteins of chromatin from different animal tissues. *FEBS Lett.* **5**, 37–40 (1969).
18. Patel, G. L., and Thomas, T. L. Some binding parameters of chromatin acidic proteins with high affinity for deoxyribonucleic acid. *Proc. Nat. Acad. Sci. USA* **70**, 2524–2528 (1973).
19. Gilmour, R. S., and Paul, J. RNA transcribed from reconstituted nucleo-protein is similar to natural RNA. *J. Mol. Biol.* **40**, 137–139 (1969).
20. Spelsberg, T. C., and Hnilica, L. S. Deoxyribonucleoproteins and the tissue-specific restriction of the deoxyribonucleic acid in chromatin. *Biochem. J.* **120**, 435–437 (1970).
21. Wang, T. Y. Tissue specificity of non-histone chromosomal proteins. *Exp. Cell Res.* **69**, 217–219 (1971).
22. Rickwood, D., Threlfall, G., MacGillivray, A. J., Paul, J., and Riches, P. Studies on the phosphorylation of chromatin non-histone proteins and their effect on deoxyribonucleic acid transcription. *Biochem. J.* **129**, 50–51 (1972).
23. Stein, G. S., and Farber, J. Role of non-histone chromosomal proteins in the restriction of mitotic chromatin template activity. *Proc. Nat. Acad. Sci. USA* **69**, 2918–2921 (1972).
24. Shea, M., and Kleinsmith, L. J. Template-specific stimulation of RNA synthesis by phosphorylated non-histone chromatin proteins. *Biochem. Biophys. Res. Commun.* **50**, 473–477 (1973).
25. Yasmineh, W. G., and Yunis, J. J. Localization of mouse satellite DNA in constitutive heterochromatin. *Exp. Cell Res.* **59**, 69–75 (1970).
26. Spelsberg, T. C., Steggles, D. W., Chytii, F., and O'Malley, B. W. Progesterone-binding components of chick oviduct. V. Exchange of progesterone-binding capacity from target to non-target tissue chromatin. *J. Biol. Chem.* **247**, 1368–1374 (1972).
27. Levy, S., Simpson, R. T., and Sober, H. A., Fractionation of chromatin components. *Biochemistry* **11**, 1547–1553 (1972).
28. Ward, S., Wilson, D., and Gillian, J., Methods for fractionation and scintillation counting of radioisotope-labeled polyacrylamide gels. *Anal. Biochem.* **38**, 90–97 (1970).
29. Weber, K., and Osborn, M., The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**, 4406–4412 (1969).
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275 (1951).
31. Burton, K., A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *J. Biol. Chem.* 62, 315–323 (1956).

32. Moore, S., and Stein, W. H., Chromatographic determination of amino acids by the use of automatic recording equipment. In “Methods in Enzymology” (S. P. Colowick and N. O. Kaplan, Eds.), Vol. 2, p. 819. Academic Press, New York, 1963.

33. Huang, R. C. C., and Huang, P. C., Effect of protein-bound RNA associated with chick embryo chromatin on template specificity of the chromatin. *J. Mol. Biol.* 39, 365–378 (1969).

34. Crampton, C. F., Moore, S., and Stein, W. H., Chromatographic fractionation of calf thymus histone. *J. Biol. Chem.* 215, 787–801 (1955).

35. Wang, T. Y., The isolation, properties, and possible functions of chromatin acidic proteins. *J. Biol. Chem.* 242, 1220–1226 (1967).

36. Benjamin, W., and Gellhorn, A., Acidic proteins of mammalian nuclei: isolation and characterization. *Proc. Nat. Acad. Sci. USA* 59, 262–268 (1968).

37. Marushige, K., Brutlag, D., and Bonner, J., Properties of chromosome nonhistone protein of rat liver. *Biochemistry* 7, 3149–3155 (1968).

38. Shelton, K. R., and Allfrey, V. G., Selective synthesis of a nuclear acidic protein in liver cells stimulated by cortisol. *Nature (London)* 228, 132–133 (1970).

39. Shaw, L. M. J., and Huang, R. C. C., A description of two procedures which avoid the use of extreme pH conditions for the resolution of components isolated from chromatin prepared from pig cerebellar and pituitary nuclei. *Biochemistry* 9, 4530–4540 (1970).

40. Rovera, G., and Baserga, R., Early changes in the synthesis of acidic nuclear proteins in human diploid fibroblasts stimulated to synthesize DNA by changing the medium. *J. Cell. Physiol.* 77, 201–212 (1971).

41. Johnson, E. M., and Allfrey, V. G., Differential effects of cyclic adenosine-3′,5′-monophosphate on phosphorylation of rat liver. *Arch. Biochem. Biophys.* 152, 786–794 (1972).

42. Stein, G. S., and Borun, T. W., The synthesis of acidic chromosomal proteins during the cell cycle of *HeLa* S-3 cells. I. The accelerated accumulation of acidic residual nuclear protein before the initiation of DNA replication. *J. Cell Biol.* 52, 292–307 (1972).

43. Le Stourgeon, W. M., and Rusch, H. P., Localization of nucleolar and chromatin residual acidic protein changes during differentiation in *Physarum polycephalum*. *Arch. Biochem. Biophys.* 155, 144–158 (1973).

44. Platz, R. D., Kish, V. M., and Kleinsmith, L. J., Tissue specificity of non-histone chromatin phosphoproteins. *FEBS Lett.* 12, 38–40 (1970).

45. Platz, R. D., and Hnilica, L. S., Phosphorylation of nonhistone chromatin proteins during sea urchin development. *Biochem. Biophys. Res. Commun.* 54, 222–227 (1973).

46. Levy, R., Levy, S., Rosenberg, S. A., and Simpson, R. T., Selective stimulation of nonhistone chromatin protein synthesis on lymphoid cells by phytohemagglutinin. *Biochemistry* 12, 224–228 (1973).

47. Gerrard, W. T., and Bonner, J., Changes in chromatin proteins during liver regeneration. *J. Biol. Chem.* 249, 5570–5579 (1974).

48. Spelsberg, T. C., Steggles, A. W., and O'Malley, B. W., Progesterone-binding components of chick oviduct III. Chromatin acceptor sites. *J. Biol. Chem.* 246, 4188–4197 (1971).

49. Spelsberg, T. C., and Hnilica, L. S., Proteins of chromatin in template restriction I. RNA synthesis in vitro. *Biochim. Biophys. Acta* 228, 202–211 (1971).

50. Augenlicht, L. H., and Baserga, R. 1973. Preparation and partial fractionation of nonhistone chromosomal proteins from human diploid fibroblasts. *Arch. Biochem. Biophys.* 158, 89–96 (1973).

51. Gilbert, W., and Müller-Hill, B., Isolation of the lac repressor. *Proc. Nat. Acad. Sci. USA* 56, 1891–1898 (1966).

52. Gilbert, W., and Müller-Hill, B. The lac operator of DNA. *Proc. Nat. Acad. Sci. USA* 58, 2415–2421 (1967).

53. Gilbert, W., and Müller-Hill, B. The lactose repressor. In “The Lactose Operon” (J. R. Beckwith and D. Zipser, Eds.), p. 93. Cold Spring Harbor Laboratory, New York, 1970.

54. Pederson, T., Gene activation in eukaryotes: Are nuclear acidic proteins the cause or the effect. *Proc. Nat. Acad. Sci. USA* 71, 617–621 (1974).

55. Riggs, A. D., Suzuki, H., and Bourgeois, S., Lac repressor operator interaction. *J. Mol. Biol.* 48, 67–83 (1970).