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Methods for Fractionation and Identification of Nonhistone Proteins of Rat Liver Chromatin

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Combinations of methods were devised for fractionation and characterization of proteins obtained from rat liver chromatin. The chromosomal proteins were extracted from DNA by treatment with 3 M NaCl, 7 M urea, or by heating in 1% sodium dodecyl sulfate. The proteins dissociatdd by sodium dodecyl sulfate were fractionated by gel filtration in the presence of the detergent. A number of discrete components, each containing a population of polypeptides of limited heterogeneity with respect to molecular weight, were isolated by this method. Proteins obtained by urea-salt dissociation were initially subfractionated into histone and nonhistone components. The nonhistone proteins were examined by sodium dodecyl polyacrylamide gel electrophoresis and by analytical and preparative isoelectric focusing methods, in an effort to provide reproducible methods for identification of specific families of proteins.

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INTRODUCTION

Unlike the histones[1,2], the nonhistone proteins of chromatin preparations are quite heterogeneous and exhibit elements of tissue and species specificity[3~5]. It has been assumed therefore, that some of the nonhistone proteins may control transcription in eukaryotic cells[6~11]. Although structural, enzymatic and regulatory roles are presumed, no specific regulatory function has been ascribed to any particular nonhistone protein component. The functional roles remain obscure partly because isolation, purification and characterization of specific components of this heterogeneous class of represent formidable experimental tasks. In the present report, methods that may conveniently be used to fractionate and purify families of nonhistone proteins are described and potential means of reproducibly identifying specific nonhistone components are presented.

MATERIALS AND METHODS

1. Preparation of nuclei. Nuclei were isolated from livers of 200~250 g male Sprague Dawley rats by methods previously described[12,13]. In brief, the livers were removed quickly and perfused with several volumes of ice cold solutions of 0.25 M sucrose in TKM buffer (0.05 M-Tirs-HCl pH 7.5; 0.025 M KCl; and 0.005 M $MgCl_2$ at 20°C). Livers were blotted, weighed, minced with scissors, added to two volumes of 0.25 M sucrose in TKM at 0°C, and homogenized in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle (clearance 0.025 cm, 15 strokes at 17,000 rev/min). The homogenate was filtered through several layers of cheesecloth and 10 m*l* aliquots were pipetted into centrifuge tubes of a SW 27 Spinco rotor. 20 m*l* of 2.3 M sucrose in TKM was then added to each tube and mixed with the 0.25 M

sucorse TKM homogenate so that the final sucrose concentration of the homogenate was 1.62 M. The mixture was underlaid with 8.5 m*l* of 2.3 M sucrose in TKM buffer using a syringe and 13-gauge needle, and as the higher density sucrose solution was introduced, the lighter homogenate was upward.

After centrifugation for 2 hrs at 25,000 RPM in a Spinco SW 27 rotor(81,500 xg) at 4°C, the supernatant was poured off. The clear white nuclear pellet was suspended in 0.25 M sucrose in TKM, sedimented at 1,000 ×g and washed twice with this buffer and twice with 0.05 M tris-HCl pH 8.0 (1,000×g centrifugation for 10 min after each wash). Nuclei were occasionally stored in 2 volumes of glycerol at −20°C.

2. Preparation of chromatin[14,15]. Washed nuclei were disrupted in 50 m*l* of 0.01 M Tris-HCl, pH 8.0 by five strokes of tight-fitting pestle in a Dounce-type homogenizer. 8.5 m*l* aliquots of this suspension were layered on 30 m*l* portions of 1.7 M sucrose (0.01 M Tris-HCl buffer, pH 8.0) in centrifuge tubes. The upper two-thirds volume of the tubes were then gently mixed and centrifuged at 25,000 RPM for 3 hrs in a Spinco SW 27 rotor at 4°C. The chromatin suspension was recovered and resuspended in 0.15 M NaCl, 0.015 M sodium citrate buffer pH 7.0 (SSC) and dialyzed against the same buffer overnight. For preparation of sheared chromatin the dialyzed suspension was stirred in a Virtis homogenizer for 90 sec at 25 volts, and then centrifuged at 1,000×g for 30 min. The supernatant is referred to as "sheared chromatin."

3. Dissociation and Fractionation of Chromosomal Proteins. Two independent methods were employed to dissociate the chromosomal proteins from DNA. In this initial study efforts were made to extract all of the protein from DNA.

(1) Purified chromatin was incubated in 1% sodium dodecyl sulfate (lauryl sodium sulfate from Schwarz Mann, Orangeburg, N. Y.) 0.01 M Tris-HCl, 10^{-3}M mercaptoethanol, pH 8, and heated to 80° for 3 min. DNA was pelleted by centrifugation at 102,000×g for 48 hrs and the supernatant was isolated. This preparations is referred to as the "SDS dissociated" protein.

(2) Alternatively, chromatin (DNA concentration 0.2～0.3 mg/m*l*) was treated with 3 M NaCl 7 M urea (ultrapure grade, Schwarz Mann) in 0.01 M Tris-HCl buffer pH 8.3 and DNA was pelleted by centrifugation at 102,000×g for 48 hrs. The supernatant referred to as the "urea-salt dissociated" chromosomal proteins.

For separation of histones from nonhistone proteins the latter "urea-salt" components were used. This supernatant was dialyzed exhaustively against large volumes of 7 M urea, in 0.01 M Tris-HCl buffer pH 8.3, to remove the salt for subsequemt fractionation into histone and nonhistone protions. After dialysis the protein component was added to QAE-Sephadex (A-50) (Pharmacia, Uppsala, Sweden) which was equilibrated with 7 M urea, 0.01 M Tris-HCl pH 8.3, (2 g Sephadex per 50 m*l* of protein solution[16]). The gel suspension was filtered and the histone components were collected in the filtrate. The gel was washed several times and the nonhistone proteins were eluted from the QAE-Sephadex with 3 M NaCl, 7 M urea, in 0.01 M Tris-HCl buffer, pH 8.3. The nonhistone proteins thus obtained were again dialyzed against 7 M urea to remove salt which would interfere with electrophoretic and isoelectric focusing results. Protein solutions were concentrated by ultrafiltration using Amicon UM-2 membranes.

4. SDS Polyacrylamide gel electrophoresis. Two different methods were used to prepare SDS gels. The first method involved gels containing 1.0% Sodium dodecyl sulfate in Tris-acetate buffer pH 7.4 according to the procedure of Fairbanks *et al.*[17] Alternatively, gels were prepared with 0.1% sodium dodecyl sulfate in Tris-glycine buffer pH 8.3 according to the procedure of Maizel[18]. Protein samples were incubated at 80° for 3 min. in 1% sodium dodecyl sulfate solution in the presence of $10^{-3}\mu$ mercaptoethanol. Gels were stained with Coomassie brilliant blue and destained by the stepwise procedure described in reference 17. Densitometric tracings of the gels were carried out using a Gilford Spectrophotometer with a Model 2410 linear transport.

5. Isoelectric focusing. Analytical gel electrocusing of the concentrated nonhistone and histone proteins was carried out in polyacrylamide gels (4% acrylamide with 0.8% NN' methylene bis acrylamide as a cross-linking agent) using 2% (W/v) pH 3～10, or pH 5～8 Ampholine, in the presence of 7 M urea. An apparatus obtained from Medical Research Apparatus (MRA), Boston, Mass. was used. Sample solutions were preincubated in 7 M urea and 5 mM dithiothreitol and focusing was carried out at constant voltage (400 V) for over 5 hours.

Preparative isoelectric focusing in sucrose density gradients was carried out in presence of 7 M urea and 5 mM dithiothreitol. The nonhistone protein components were fractionated by preparative isoelectric focusing in a 0～50% sucrose gradient. An LKB model 8101 column of 120 m*l* capacity was used for these experiments. Ampholine pH 3～10 at final concentrations of 1% was used to establish a pH gradient. Isoelectric focusing was performed for 72 hrs at 1100 V and samples were eluted from the bottom of the column at the conclusion of the focusing. The pH of each fraction was measured using a Beckmann 1019 pH meter. In spite of trace amounts of isocyanate often found in urea preparations, there was no evidence for carbamylation using several control proteins exposed to the urea under these conditions.

RESULTS AND DISCUSSION

The "SDS dissociated" chromosomal proteins were fractionated by chromatography on Bio-gel A 1.5 m columns equilibrated in the presence of 1% sodium dodecyl sulfate. A typical elution pattern is shown in Fig. 1. Essentially all of the protein applied to the column was recovered in the eluate. The highest molecular weight component (A) was a mixture of protein free DNA with trace amounts of high molecular weight polypeptides coeluting with DNA. The other fractions (B–I) consisted predominately of protein with molecular weights in the range of about 150,000 to 10,000. Approximate molecular weight ranges found were: A, > 150,000; B, 130～150,000; C, 90～120,000; D, 70～90,000; E, 55～70,000; F, 40～50,000; G, 20～250,000; H, 10～20,000; I, 10,000 and 45,000. The elution pattern for sheared chromatin samples or samples digested by endogenous nucleases was more complex since DNA fragments coeluted with the proteins.

Analytical sodium dodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns for some of the Bio-gel A 1.5 m components are shown in Fig. 2, and it is evident that each component consists of a population of polypeptides of limited heterogeneity with respect to molecular weight. Component F included histone F1 and component H contained the other histones. On the basis of its molecular weight, histone F1 had anomolous electrophoretic

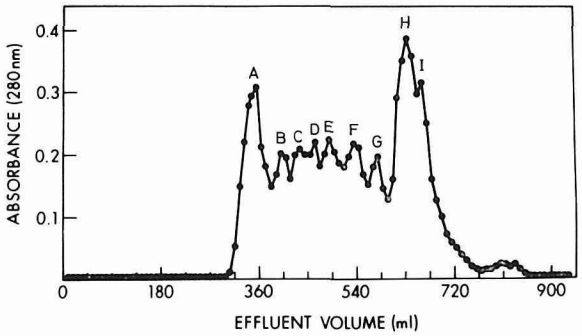

Fig. 1 Fractionation of chromosomal proteins in 1% sodium dodecyl sulfate on aBio Gel A 1.5 m column. Bio Gel A 1.5 m (200–400 mesh) was washed several times with 0.1 M Tris-HCl pH 8 and equilibrated with 1% sodium dodecyl sulfate 0.1 M Tris HCl buffer, pH 8. The column (180×2.5 cm) was filled at room temperature with Bio Gel A 1.5 m in the presence of 1% sodium dodecyl sulfate, 0.1 M Tris HCl, pH 8 with descending flow controlled at less than 10 m*l*/hr. After packing of the column, the flow rates were adjusted by hydrostatic pressure to about 15 m*l*/hr flow rate and the column washed for 24 hr with this buffer. Samples containing 20 mg of chromatin protein were applied to the column chromatography was carried out at room temperature with a flow rate of 15 m*l*/hr maintained throughout the elution. Fractions of 5 m*l* volume each were collected, and absorbance at 280 nm of each fraction measured using a Cary 14 spectrophotometer.

mobility in SDS gels and also eluted as an apparently higher molecular weight component from the SDS column. Comparisons between two analytical SDS gel systems (trisacetate and tris-glycine, Fig. 2A and 2B), show that the patterns are variable and are dependent upon electrophoretic conditions. It samples were incubated in the same way prior electrophoresis to (80° in 10^{-3}M mercaptoethanol, 1% sodium dodecyl sulfate) some components showed a tendency to form aggregates in gels prepared in 0.1% SDS (Fig. 2B, tris-glycine system) whereas the same components did not aggregate during electrophoresis in the tris acetate system (Fig. 2A). The lowest molecular weight component I (MW 10,000) however exhibited a band of higher molecular weight (42～45,000) in both gel systems.

The "urea-salt" dissociated chromosomal proteins were divided into histone or basic protein and nonhistone protein fractions, and were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing. Densitomentric scans of sodium dodecyl sulfate-gels of the histone and nonhistone components are shown in Fig. 3. The lower molecular weight components consisted mainly of the more basic proteins and histones, and the majority of nonhistone proteins had molecular weights greater than 30,000. It is evident, however, that some basic nonhistone proteins accompanied the histones in this fractionation. On the other hand, the results in Figs. 2 and 3 show that the nonhistone fraction is free of histone contamination.

The nonhistone proteins were resolved into discrete components by analytical gel isoelectric focusing. Figure 4 shows that the more acidic nonhistone components are easily distinguished from the histones and more basic nonhistone proteins. Large scale quantities of the non-

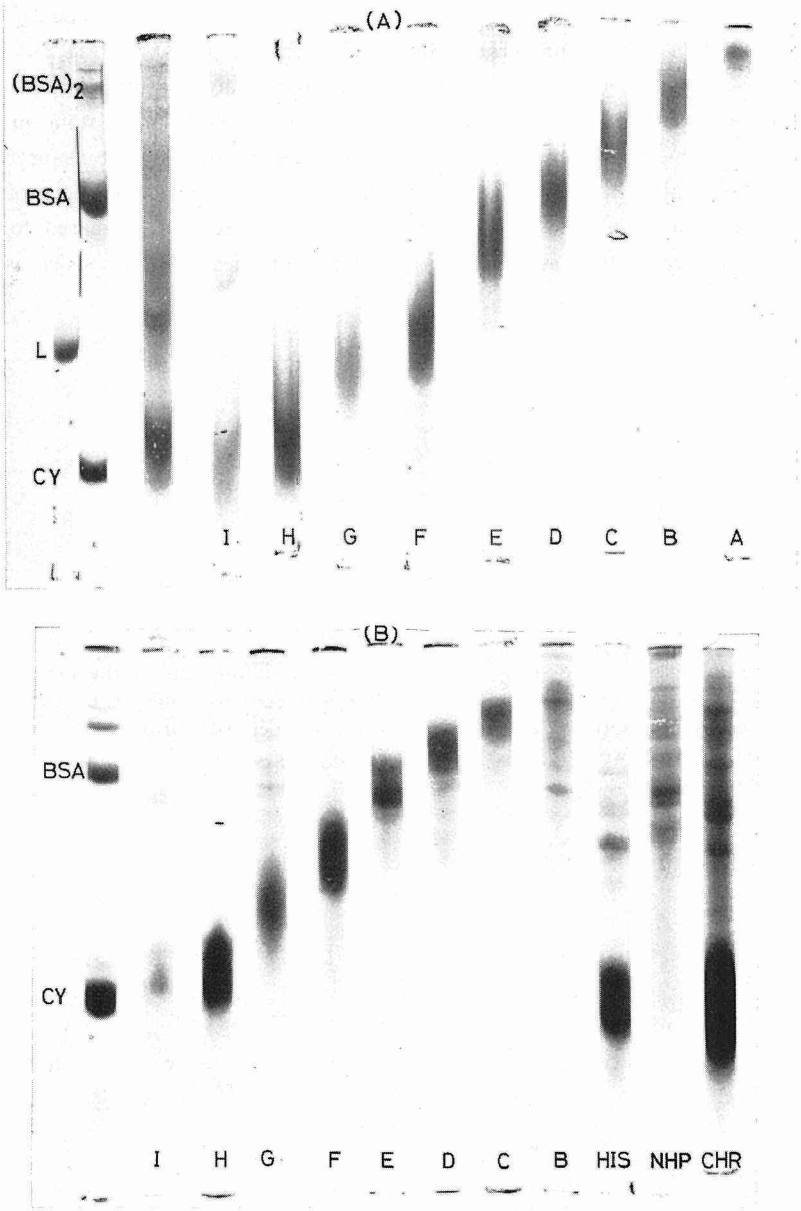

Fig. 2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of chromosomal protein components isolated by gel filtration on Bio Gel A 1.5 m (see Fig. 1). Componets A–I refer to corresponding fractions in Figure 1.

Protein samples were preincubated for 3 min at 80° in 1% sodium dodecyl sulfate 10^{-3} M mercaptoethanol, and electrophoresis was carried out in 10% polpacrylamide gels. The top series of gels (A), were prepared according to the method of Fairbanks *et al.*[17] in 1% sodium dodecyl sulfate and the bottom series of gels (B) were prepared in a sodium dodecyl sulfate Tris-glycine system.[18] Molecular weight markers indicated are (BSA) 2-bovine serum albumin dimer, BSA- bovine serum albumin (MW 69,000), L-ligandin (MW 23,000) and CY-cytochrome-c (MW 12,000). CHR-is unfractionated chromatin, HIS are histones, and NHP are nonhistone protein components obtained by QAE- Sephadex fractionation, as described in the text.

histone protein components were also fractionated by preparative isoelectric focusing procedures and an elution pattern from a preparative focusing column is shown in Fig. 5. The shape of this elution profile is comparable to that obtained on analytical focusing gels shown in Fig. 4. The results in Fig. 5 shown that the great majority of the nonhistone proteins have isoelectric points in the pH range of 6～8, and in this regard the nonhistone proteins should not be considered highly acidic proteins. Although the amino acid compositions of these proteins generally show a large excess of glutamic and aspartic acid as compared to basic residues,[19~21] it is conceivable that an appreciable number of these residues exist as glutamine

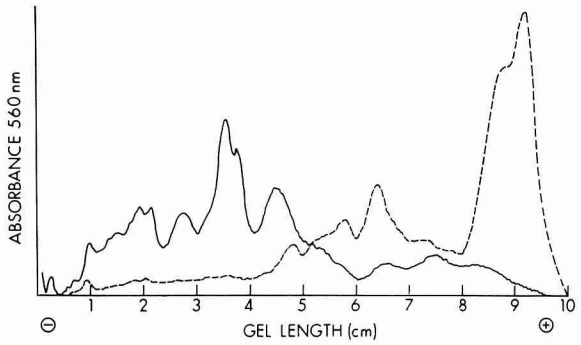

Fig. 3 Densitometric scans of sodium dodecyl sulfate gels of the histone (dotted line) and nonhistone (solid line) components obtained by QAE-Sephadex fractionation of the urea-salt dissociated chromosomal proteins. Gels were 10% acrylamide prepared according to the method of Fairbanks *et al.*[17]

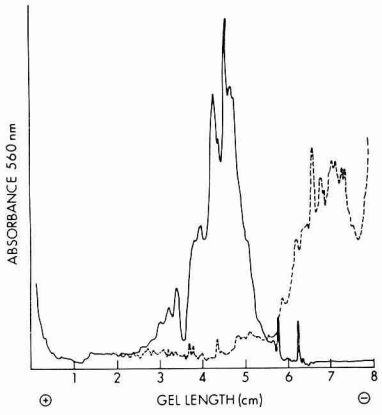

Fig. 4 Gel isoelectric focusing of the histone or basic protein and nonhistone components of rat liver chromatin. The histones (dotted line) and nonhistones (solid line) are shown in the densitometric scan. The gel was prepared in the presence of 7 M urea according to the procedure outlined in the text, using. pH 3.5–10 ampholines.

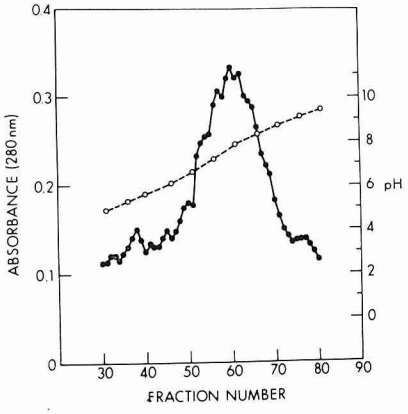

Fig. 5 Preparative column isoelectric focusing profile of nonhistone protein components obtained after QAE- Sephadex fractionation of the chromosomal proteins. Ampholine (1%), pH 3.5–10 was used and focusing carried out for 72 hrs at 1100 V in the presence of 7 M urea 5 mM Dithiothreitol in a 0–50% sucrose gradient.

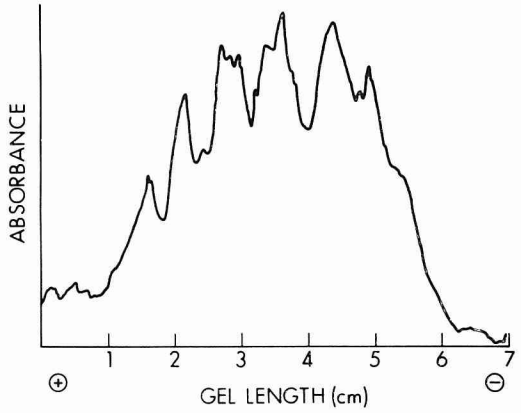

Fig. 6 Analytical gel isoelectric focusing of the nonhistone proteins. Focusing was carried out in the presence of 7 M urea using 1% carrier ampholine, pH 5–8.

and asparagine in the native proteins. It is noteworthy that our preparations were not treated with mineral acid or other extreme conditions to extract histones, and thus extensive deamidations may have been avoided.

Higher resolution of the nonhistone components could be attained by isoelectric focusing over a narrower pH range of 5～8. A typical analytical gel focusing pattern in this pH range is shown in Fig. 6, and at least 22 major polypeptide bands were observed. The methods described in this report thus present useful means for preparation, fractionation and characterization of the major chromosomal proteins from rat liver. Differences in size and charge of consitituent polypeptides and combinations of these two properties from a simple basis for their separations.

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