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Note

Analysis and preparation of chromosomal high-mobility group proteins by ion-exchange high-performance liquid chromatography

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High-mobility group (HMG) proteins represent a group of major chromosomal non-histone proteins (for a review see ref. 1). Four main HMG proteins have been characterized in calf thymus, HMG 1, 2, 14 and 17, and have been separated by acid-urea polyacrylamide gel electrophoresis [2, 3]. These proteins have been found in many species [4], with tissue-specific variations [5]. They are characterized by their solubility in 5% perchloric acid, by their high electrophoretic mobility and by their amino acid composition (ca. 50% charged amino acids, a small number of aromatic amino acids, absence of cystein).

HMG proteins remain relatively unchanged, as do histones, during their evolution. Their biological role is not fully understood at the present time. HMG 1 has been shown to destabilize DNA double helix and to bind to single-stranded DNA [6]; it is a physiological nucleosome assembly factor [7]. HMG 1 and 2 seem to be required for the transcription of chromatin [8]. HMG 14 and 17 are bound to nucleosomes [9], and their association with actively transcribed genes is controversial [10, 11].

Several minor HMG proteins have been described: HMG 18 and 19 in calf thymus and liver [12], AP-X and AP-Y in calf thymus [13], HMG I and M proteins in HeLa cells [14]. In a preliminary work we recently found two new HMG proteins, HMG O and HMG X, with tissue-specific and cell growth variations [15].

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In this work we describe a very simple and rapid procedure using ion-exchange high-performance liquid chromatography (HPLC) for semi-quantitative analysis and preparation of HMG O, X, 14 and 17.

EXPERIMENTAL

Preparation of the perchloric-acid-soluble material

Livers from Wistar rats weighing 180—200 g, fasted for 36 h, were minced and homogenized with a Thomas Potter homogenizer in 5% perchloric acid and 0.5 m*M* phenylmethylsulphonyl fluoride (0.5 ml/g of liver) (Boehringer, Mannheim, F.R.G.), at 4°C. The homogenate was centrifuged in a Sorvall RC 2B centrifuge at 15 000 *g* for 30 min at 4°C. The clear yellow supernatant was extensively dialysed against 1 m*M* acetate buffer (pH 5.0) and lyophilized. It was dissolved in buffer A: 0.04 *M* phosphate buffer (pH 4.4), 0.1% Brij 35. All chemicals were of the purest grade from Merck (Darmstadt, F.R.G.).

Ion-exchange high-performance liquid chromatography

A Chromatem 800 HPLC system (Touzart et Matignon, Vitry, France) was used with a 5 × 0.5 cm prepacked Mono S monodispersed cation-exchange column, particle size 10 µm (Pharmacia, Uppsala, Sweden). Experiments were conducted at 20°C. The non-linear gradient shown in Fig. 1 was carried out using an Apple II computer. Elution was obtained by increasing the ionic strength. The starting buffer was buffer A. The high-ionic-strength buffer was the same but supplemented with 1 *M* sodium chloride. The flow-rate was 1 ml/min, and 0.5-ml fractions were collected. Optical density was monitored at 220 nm using a Shimadzu spectrophotometer.

Polyacrylamide slab gel electrophoresis

SDS—polyacrylamide gel. The electrophoreses were performed according to Laemmli [16] with a 7.5—20% acrylamide gradient and a 4% stacking gel.

Acid-urea polyacrylamide gel. The electrophoreses were performed according to Panyim et al. [17] with 15% acrylamide, modified for slab gel by the addition of a 7.5% acrylamide stacking gel. All gels were stained with Coomassie Brilliant Blue R 250 (Bio-Rad Labs., Richmond, CA, U.S.A.).

Protein determination

Protein amounts were estimated by direct weighing of lyophilized samples since it was not possible to use spectrophotometric methods because of the small number of aromatic residues. For small amounts, proteins were estimated using the Bio-Rad assay kit.

Amino acid analysis

The samples were hydrolysed in 5.7 *M* hydrochloric acid at 120°C in a vacuum for three different periods of time from 24 to 72 h. The amino acids were separated and determined with an automatic Chromospeck J 180 amino acid analyser (Rank Hilger, Margate, U.K.).

RESULTS

Preparative chromatography

Large-scale preparation (Fig. 1A). The perchloric-acid-soluble material was lyophilized, 80 mg of protein were dissolved in 0.4 ml of buffer A and transferred to a prepacked cation-exchange Mono S column. The flow-rate was 1 ml/min. Under these conditions, 1—1.2 mg of HMG O were obtained in pure form, as shown with electrophoresis performed in SDS—polyacrylamide gel which was overloaded (Fig. 2D). HMG X and 14 were relatively poorly separated but they may be obtained in a pure form either by taking the top of each peak or by a second chromatography on the same column using an extended 0.3—0.8 *M* sodium chloride gradient. HMG 17 was obtained in a pure form as a large peak. Histones 1A and 1B were eluted together in a single peak, as were HMG 1 and 2.

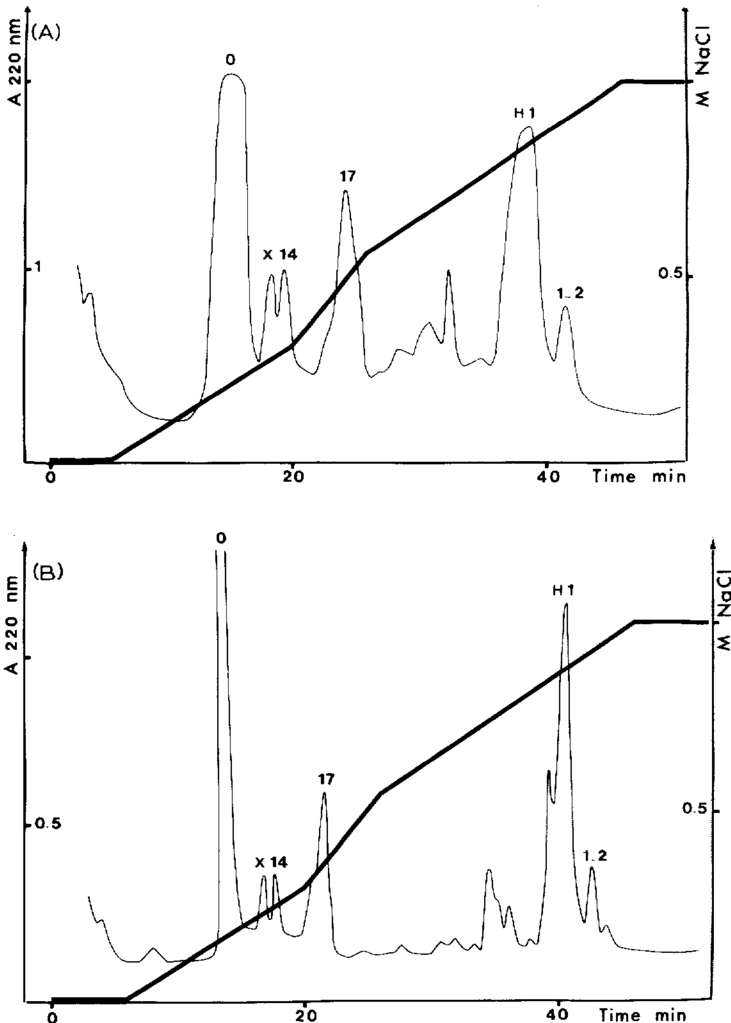

Fig. 1.

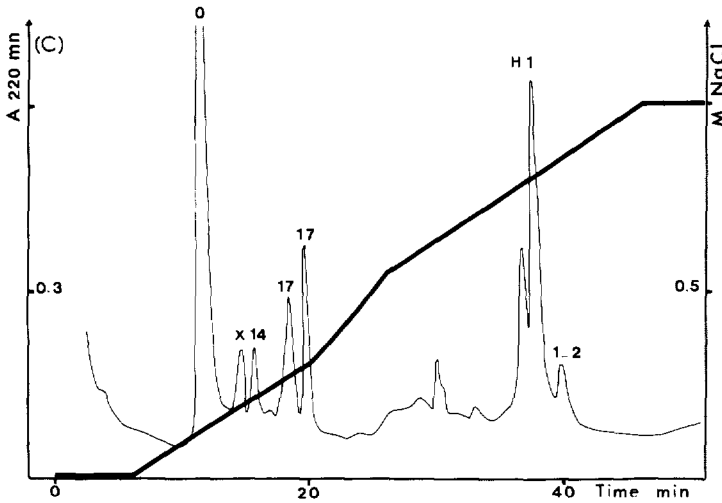

Fig. 1. Preparation and analysis of HMG proteins by ion-exchange HPLC. Chromatography was performed with a Chromatem 800 HPLC system using a 5 × 0.5 cm Mono S cation-exchange column. The starting buffer was 0.04 *M* phosphate buffer (pH 4.4), 0.1% Brij 35. A linear sodium chloride gradient was used for the separation. (A) Large-scale preparation: 80 mg of lyophilized protein from 5% perchloric acid extract, dissolved in 4.0 ml of phosphate buffer were used. (B) Small-scale preparation: the same fractionation was performed with 5 mg of protein dissolved in 0.15 ml of phosphate buffer. (C) Analytical study; the same experiment was performed with 200 μg of protein dissolved in 20 μl.

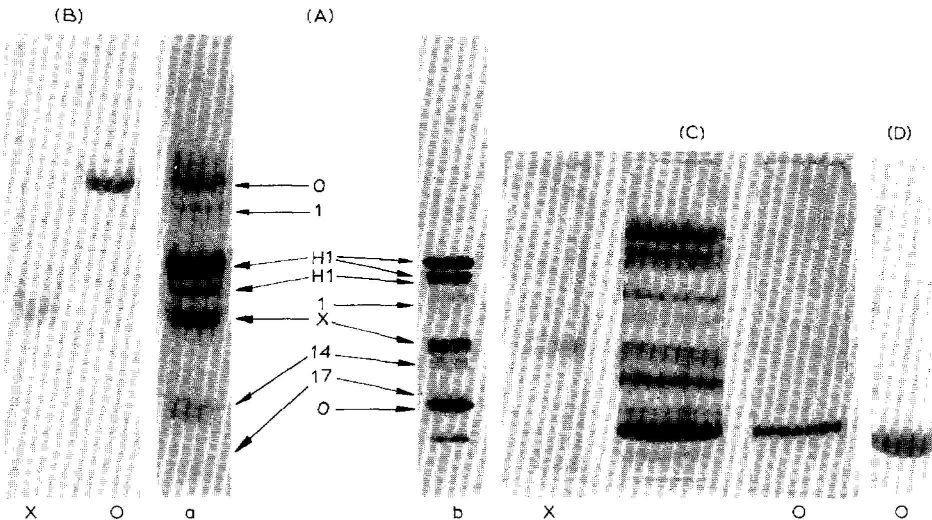

Fig. 2. Polyacrylamide gel electrophoresis of HMG proteins. (A) Perchloric acid extract of rat liver: (a) acid-urea polyacrylamide gel [17]; (b) SDS—polyacrylamide gel [16]. (B) Acid-urea polyacrylamide gel of HMG O and X obtained by small-scale preparative chromatography (see Fig. 1B). (C) SDS—polyacrylamide gel of HMG O and X obtained by small-scale preparative chromatography (see Fig. 1B) and of perchloric acid extract. (D) SDS—polyacrylamide gel of HMG O (30 μg) obtained by large-scale preparative chromatography (see Fig. 1A).

Small-scale preparation (Fig. 1B). The same experiment was performed with 5 mg of perchloric-acid-soluble protein. This technique allows the preparation, in a pure form, in a single step of 40 min of HMG O, X, 14 and 17 (ca. 10–40 μg of each). The purity of the two new HMG proteins, O and X, was controlled by electrophoresis on acid-urea polyacrylamide gel (Fig. 2B) and in SDS–polyacrylamide gel (Fig. 2C).

Analytical chromatography (Fig. 1C). The same experiment was performed with 200 μg of perchloric-acid-soluble protein. All the HMG proteins, except 1 and 2 which were eluted in a single peak, were well separated. HMG 17 was eluted in two peaks. Each peak was submitted to a SDS–polyacrylamide gel electrophoresis, and they migrated as a single peak with identical mobility. It may be suggested that one of these peaks corresponds to a phosphorylated HMG [18]. Our attempts to demonstrate it by electrophoresis were unsuccessful: HMG 17 migrated too fast in an acid-urea gel to allow sufficient resolution; the use of immobiline, which is very resolutive, was impossible because of a strong interaction between the HMG proteins and the charged matrix [19].

Amino acid composition (Table I)

The amino acid composition of HMG 14, 17, O and X was determined on proteins prepared by the small-scale chromatographic method. The compositions found for HMG 14 and 17 are in agreement with the data obtained by others [20].

TABLE I

AMINO ACID COMPOSITION OF HMG O, X, 14 and 17

The proteins were obtained by the small-scale preparation procedure. The proteins were hydrolysed in 5.7 *M* hydrochloric acid at 120°C for three different periods of time. The amino acids were separated and determined with an automatic Chromospeak J 180 amino acid analyser.

Amino acid (%)	HMG O	HMG X	HMG 14	HMG 17
Aspartic acid	9	10.5	6.5	8
Threonine	4.5	3	2.5	5
Serine	5.5	5.5	3	5
Glutamic acid	12	23	14	10
Proline	8	7.5	12	6
Glycine	12	8.5	11	10
Alanine	12	9	15	8
Half-cystine	0	0	0	0
Valine	5	4	1	4
Methionine	1	0.5	0	0
Isoleucine	6.5	1	0	1
Leucine	5.5	3	3	4
Tyrosine	3	1	1	1
Phenylalanine	3	1	1	2
Histidine	1	1	0	1.5
Lysine	9.5	14	22	19
Arginine	3	4.5	7	9

The newly described HMG O and X present the characteristics of HMG proteins: high content of charged amino acid, low aromatic acid content, absence of cystein.

A similar amino acid composition has been obtained with HMG separated by preparative electrophoresis in acid-urea gels.

DISCUSSION

Most of the methods used for the preparation and purification of the HMG proteins are derived from the technique of Johns [1], initially used for the fractionation of histones. They involve extraction with either 5% perchloric acid or 0.35 *M* sodium chloride followed by precipitation with acetone. Fractionations of the HMG proteins were carried out on CM-Sephadex columns [3, 21, 22] at various pH values: 5—5.5 [22], 8.8 [21], 9 [3]. Fractionations were also performed by chromatography on DNA-cellulose columns [23] and by preparative electrophoresis on SDS—polyacrylamide gel [24, 10].

The presence of proteolytic enzymes in most of the tissues studied shows that the use of denaturing conditions is required as well as a rapid preparation procedure.

Most of the previous works have been carried out on nuclei or on chromatin as starting material. It has been shown [25] that these proteins are localized in cytosol as well as in nuclei, which led us to use total liver as starting material. We also suppressed the acetone precipitation step, which could discard some of these proteins.

The use of HPLC has highly improved the results by reducing the duration of the fractionation and by improving the resolution: we have observed that HMG O has a strong tendency to form aggregates and to precipitate during dialysis. We have been able to avoid loss of material by a strict pH control and by vigorous stirring during the dialysis. The use of total organs and the conditions described above could explain how we have been able to detect a new HMG protein present in relatively large amounts. However, the separation of HMG 1 and 2 from the elution peak would require an additional step.

The technique described in this paper may be used for two purposes: it represents a rapid and efficient method for the preparation, in relatively large amounts, of several individual HMG proteins; it may be used for analytical studies of HMG proteins, since it has been shown that these proteins present tissue-specific distribution [5, 26], variations according to the proliferative activity of the cells [27], and changes related to commitment of cells to differentiation [28]. This method can also be used to determine the relative amounts of histones H 1A and H 1B, which are modified during neoplastic transformation [29].

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