A METHOD FOR THE FRACTIONATION OF THE HIGH-MOBILITY-GROUP NON-HISTONE CHROMOSOMAL PROTEINS

Clive Sanders
Department of Biochemistry & Chemistry
Guy's Hospital Medical School
London, SE1 9RT

Received August 15,1977

Summary: A method is given for the preparation of four non-histone chromosomal proteins, one of which, protein 14, hitherto has not been isolated. The method also enables the preparation of histone H1 in gram quantities. The four non-histone chromosomal proteins so prepared are all polar molecules over 50% of each being composed of acidic and basic amino acids. It is also shown that protein 14 can be prepared from calf thymus without prior isolation of chromatin.

INTRODUCTION

Chromatin contains a discrete group of non-histone proteins which are characterised by their relatively low molecular weight ($<30,000$) and their high content of acidic and basic amino acids (1). These proteins, which have been designated (2) high-mobility-group (HMG) proteins, can be extracted from chromatin with either 0.35M NaCl (3) or 0.75M $HClO_4$ (4). The high-mobility-group proteins have been found in various mammalian tissues at a concentration of 10^5-10^6 molecules of each per nucleus (1) and more recently evidence has been presented which suggests that these proteins are present on monomer nucleosomes (5).

As a prerequisite for the study of these proteins, methods have been developed for the isolation of proteins 1 and 2 (6) and for protein 17 (3,7). I described here a method for the fractionation of high-mobility-group proteins which enables the isolation of the four major proteins of the group, proteins 1, 2, 14 and 17.

Abbreviations: HMG, high-mobility-group
PMSF, phenylmethyl-sulphonyl fluoride

ISSN 0006-291X

METHODS AND RESULTS

Preparation of high-mobility-group proteins from calf thymus chromatin

All operations were done at 4°C unless stated otherwise. Calf thymus was collected within one hour of the animals' death, washed with 0.05M phenylmethyl-sulphonyl fluoride (PMSF) in propan-2-ol, transported to the laboratory on ice, where the chromatin preparation was begun within 3 hours of the animals' death.

5 x 100g batches of minced calf thymus were each homogenized for 15s in a commercial Waring blender at fast speed with 600 cm^3 of 0.14M NaCl, 0.5mM PMSF. (50 mM PMSF in propan-2-ol was added to the saline just prior to homogenization.) The homogenates were centrifuged at 2,000g for 15 min, the supernatants discarded and the sediment homogenized and centrifuged as before. This process was repeated to give three washings in all, after which the sediments were combined into two lots, each of which was homogenized for 40s with 600 cm^3 of 0.14M NaCl, 0.5mM PMSF and centrifuged at 2,000g for 20 min.

The two chromatin sediments were combined and homogenized (Waring blender, 2 min, fast setting) with 300 cm^3 of 0.75M $HClO_4$. The homogenate was centrifuged at 2,000g for 30 min, the supernatant retained and the sediment homogenized in the same way with 400 cm^3 of 0.75M $HClO_4$ and centrifuged at 2000g for 20 min. The supernatants were combined and further clarified by filtering through a grade-2 followed by a grade-4 sintered-glass funnel to give a final volume of 1dm^3. To precipitate histone H1 3½ volumes of acetone were added with rapid stirring and the solution made 0.07M HCl by addition of conc. HCl. The solution was centrifuged for 15 min at 2,000g, the supernatants retained and the precipitate, histone H1, washed once in acetone/0.1M HCl (3.5:1 v/v), three times in acetone and dried under vacuum. The yield of histone H1 was 1.8 gm.

After removal of any traces of H1 precipitate by filtration of the supernatants through a grade-4 sintered-glass funnel the HMG proteins were precipitated by the addition of 2½ volumes of acetone (based on the original volume of extract). The precipitate, designated B, was collected by centrifugation at 2000g, washed three times in acetone and dried under vacuum. The yield of precipitate B, which contained the HMG proteins, was 400 mg. A schematic diagram is given in Fig. 1.

Preparation of high-mobility-group proteins from calf thymus without prior isolation of chromatin

The method is essentially that reported previously (4). All operations were carried out at 4°C.

The calf thymus used was either from animals slaughtered that day or had been stored at -20°C.

150gm of minced calf thymus was homogenized with 150 cm^3 of 1.5M $HClO_4$ for 1 min at slow speed followed by 2 min at fast speed in a commercial Waring blender. The homogenate was centrifuged for 30 min at 2000g, the supernatant retained, and the sediment homogenized with 150 cm^3 of 0.75M $HClO_4$ and centrifuged in the same way. The combined supernatants were further clarified by filtration through a grade-2 followed by a grade-4 sintered-glass funnel. 3½ volumes of acetone were then added to the filtrates and the resulting solution made 0.07M HCl by the addition of conc. HCl. The precipitate which formed consisted mainly of histone H1 and was removed by centrifugation followed by filtration of the supernatant. (It is possible to

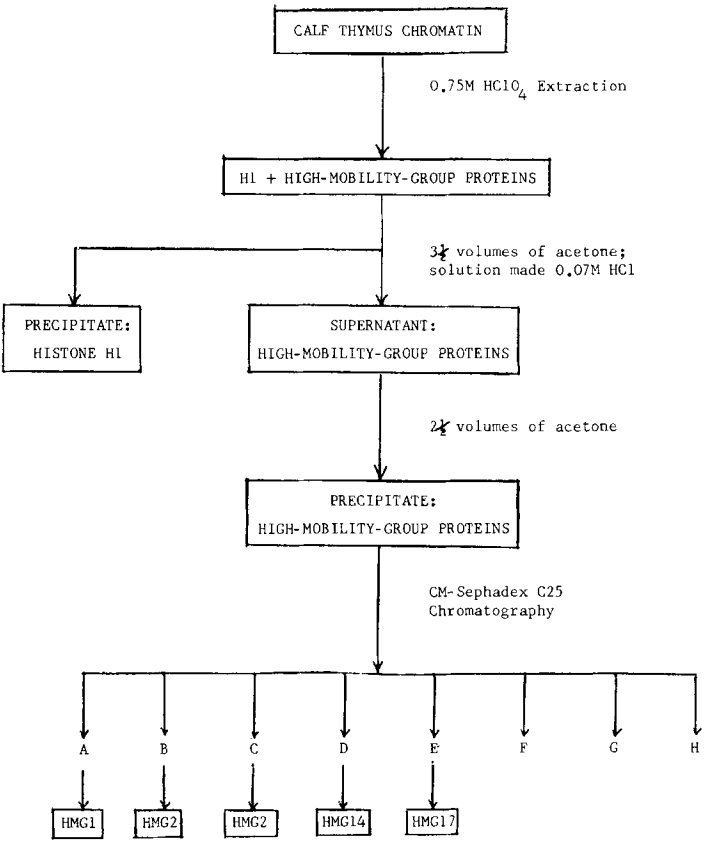

Figure 1. Schematic diagram for the fractionation of HMG proteins.

collect this histone H1 by the same procedure as described previously and the H1 so prepared is reasonably pure, giving a single band on electrophoresis of 10-20μg of protein.) To this solution 2½ volumes of acetone (based on the original volume of extract) were added and the precipitate which contained the HMG proteins, was collected by centrifugation at 2000g for 15 min, washed three times in acetone and dried under vacuum. The average amount of the precipitate isolated in this manner was 100 mg.

CM-Sephadex C25 Chromatography of High-Mobility-Group Proteins

The gradient sievorptive chromatographic principle as described by Kirkegaard (8) and employed previously by Goodwin et al, (3) for the fractionation of the HMG proteins is employed here.

360 mg of HMG protein isolated from calf thymus chromatin was dissolved in 5 cm^3 of 7.5mM sodium borate buffer (pH 9.0), 0.2M NaCl and the protein solution titrated with 1M NaOH, to pH 9.0. Sodium borate buffer (7.5mM, pH 9.0) and 1M NaCl were added to give a 10 cm^3 0.2M NaCl, 7.5mM sodium borate, pH 9.0 solution. The solution was clarified by centrifugation and applied to a 2.3 x 30 cm column of CM-Sephadex C25 equilibrated with 7.5mM sodium borate, pH 9.0m buffer. 100 cm^3 of 0.2M NaCl, 7.5mM borate, pH 9.0

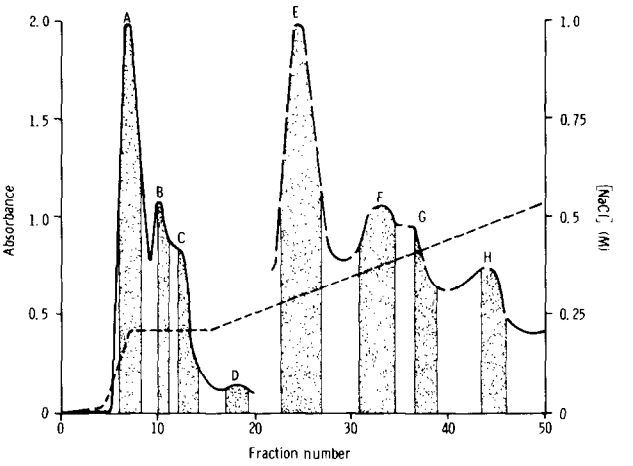

Figure 2. CM-Sephadex C25 chromatography of HMG protein. Approximately 360 mg of precipitate B was applied to a 2.3 x 30 cm column of CM-Sephadex C25, equilibrated with 7.5mM sodium borate buffer (pH 9.0) and eluted as described in Methods. 10 cm^3 fractions were collected.
————, Absorbance at 280nm; ——— ———, absorbance at 230nm; -------, NaCl concentration.

buffer was pumped through the column at a flow rate of 0.5 cm^3/min after which the sample applicator was adjusted so that it was in direct contact with the top of the CM-Sephadex C25 gel. A linear salt gradient consisting of 500 cm^3 of 0.2M NaCl, 7.5mM sodium borate buffer pH 9.0 and 500 cm^3 of 1M NaCl, 7.5mM sodium borate buffer pH 9.0 was applied to the column at the same flow rate. 10 cm^3 fractions were collected and their absorbance at 230 nm and 280 nm measured in a 1 cm cuvette. The elution profile is shown in Fig. 2. Fractions F-H were diluted to 0.35M NaCl and all the fractions were acidified to 0.1M HCl before the addition of 6 volumes of acetone to precipitate the protein. The precipitates were collected by centrifugation, washed once in acetone/0.1M HCl (6:1 v/v), then three times with acetone and dried under vacuum. The yields of fractions F, G and H were 30 mg, 17 mg and 2 mg respectively. The yields and total amino acid analysis of fractions A-E are given in Table 1. Comparative polyacrylamide gel electrophoresis of these fractions with the original unfractionated material was carried out and the results are shown in Fig. 3.

From the comparative polyacrylamide gel electrophoretic results it can be seen that fractions A, D and E correspond to HMG proteins 1, 14 and 17 respectively. Fractions B and C correspond to HMG protein 2 and probably represent different sub-fractions of this protein which is known to be microheterogeneous (9). Although no cross-contamination of the proteins was revealed by comparative gel electrophoresis, when 20μg samples of fractions A-E were analysed by single gel electrophoresis faint bands of impurity could be discerned. Sodium dodecyl sulphate-polyacrylamide gel

Table 1

The amino acid composition (Mol %) and yields of fractions (A-E) obtained by CM-sephadex C-25 chromatography of HMG proteins extracted from chromatin and of HMG 14 prepared by direct extraction of calf thymus with 0.75M $HClO_4$ followed by CM-sephadex C-25 chromatography.

AMINO ACID	A	B	C	D	E	HMG14
Asp	10.4	10.5	10.0	8.8	9.8	9.0
Thr	2.2	2.0	2.2	3.5	1.6	3.6
Ser	4.3	5.8	4.2	6.8	3.0	6.1
Glu	17.3	18.7	16.8	15.9	9.9	15.8
Pro	7.0	7.9	6.8	8.8	11.8	9.2
Gly	5.1	6.5	5.3	6.8	9.3	7.5
Ala	9.1	8.1	8.8	11.9	15.1	15.6
Cys ($\frac{1}{2}$)	0.2	0.1	0.2	-	-	-
Val	2.4	2.0	2.4	3.7	3.0	3.8
Met	2.3	2.4	2.2	1.2	0.8	1.5
Ile	2.0	1.7	1.9	1.3	1.1	0.4
Leu	2.1	2.2	2.0	2.6	1.6	1.9
Tyr	3.3	3.1	3.2	1.2	0.9	0.3
Phe	4.2	4.1	4.0	1.7	1.2	0.4
His	1.5	2.0	1.4	0.8	0.5	0.2
Lys	22.6	19.9	23.5	19.7	25.6	21.4
Arg	4.0	4.7	5.1	5.0	4.8	4.3
Yield (mg protein)	60	17	22	7	30	20

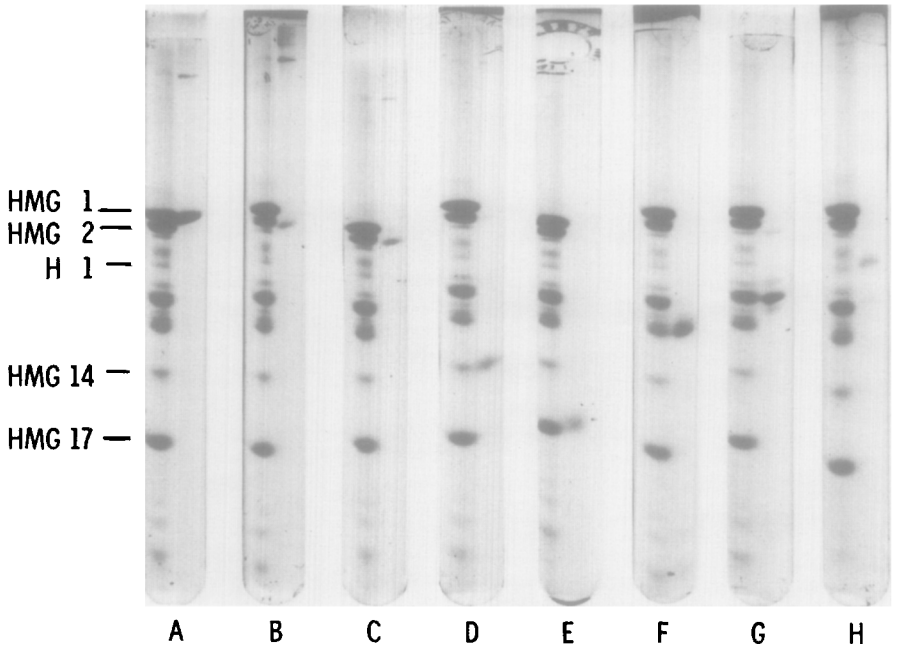

Figure 3. Comparative polyacrylamide gel electrophoretic patterns of fractions A-H obtained by CM-Sephadex C25 chromatography of HMG proteins. Right-hand side of gels shows fractions A-H as indicated, left-hand side of each gel shows the original sample which was applied to the column.

electrophoresis of HMG proteins 1, 2, 14 and 17 revealed no additional impurity; each of the protein samples migrated as a single band. The molecular weight of protein 14 was not determined since this method has been shown to give unreliable results when applied to such highly basic proteins (3, 6).

The amino acid composition of the proteins so prepared are in reasonable agreement with those published previously for proteins 1, 2 (3,6) and 17 (3,7).

Chromatography of HMG proteins extracted directly from calf thymus without prior isolation of chromatin was carried out in exactly the same way except that the sample solution was made 0.15M NaCl, 7.5mM sodium borate (pH 7.0) and the salt gradient therefore was from 0.15M NaCl to 1M NaCl. The yield and total amino acid analysis of protein 14 prepared in this way from

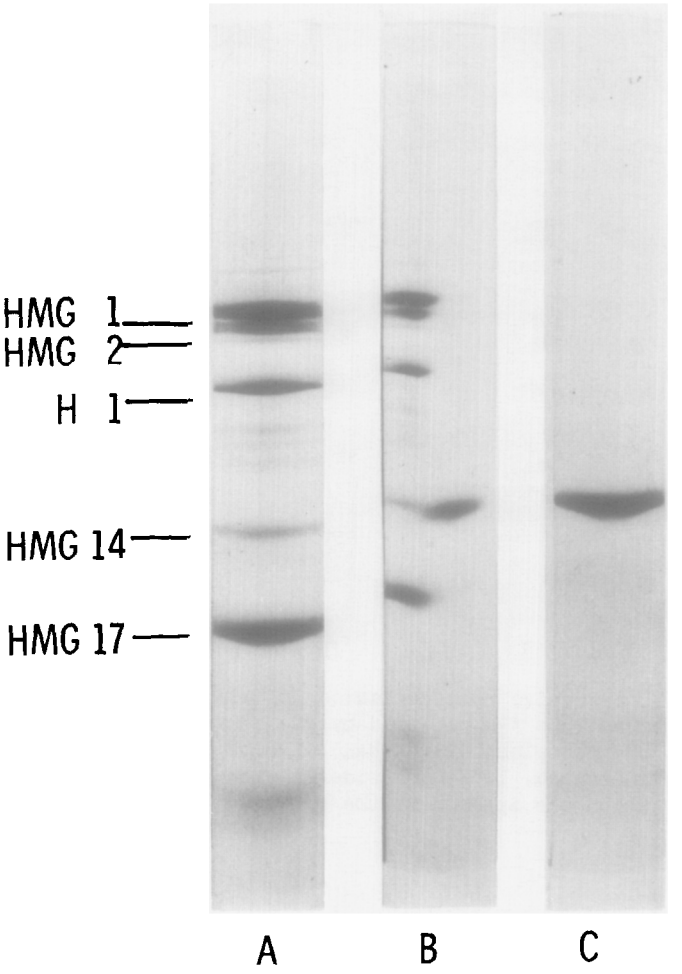

Figure 4. Comparative polyacrylamide gel electrophoresis of protein 14 prepared by chromatography of HMG protein extracted by 0.75M $HClO_4$ from calf thymus without prior isolation of chromatin.
Gel A 100μg of HMG protein
Gel B Comparison of protein 14 with HMG protein
Gel C 20μg protein 14.

lg of original material is given in Table 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of this preparation was carried out and the sample migrated as a single band. Comparative polyacrylamide gel electrophoresis of protein 14, prepared by this method, with the original unfractionated material is shown in Fig. 4 and from these results it can be

seen that this method yields a product of high purity, superior to that obtained by the previous method described above.

The analysis of protein 14 so prepared is in good agreement with that of the protein when prepared from calf thymus chromatin. The discrepancy in alanine content may be attributed to the presence of impurities, in the sample of protein 14 when it was prepared from chromatin, which are absent when the protein is prepared directly from the tissue.

Polyacrylamide gel electrophoresis

Comparative polyacrylamide gel electrophoresis of two protein samples on the same gel was carried out by the method developed by Johns (10), using 20% polyacrylamide gels (0.9 x 12 cm) at pH 2.4. Polyacrylamide gels of identical composition were also used for analysis of single samples (11). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn (12); 10% polyacrylamide gels (0.9 cm x 12 cm) were used and electrophoresis was at 18 ma per tube for $4\frac{1}{2}$h.

Amino acid analysis

Total amino acids were measured using a JEOL automatic amino acid analyser modified for single column analysis. Samples were hydrolysed for 24 hr in 6M HCl at 115°C. No corrections were made for hydrolytic losses but a crystal of phenol was added to each sample prior to hydrolysis to avoid tyrosine breakdown.

DISCUSSION

In view of the relatively low molecular weights ($< 30,000$) of the high mobility group proteins the possibility exists that they could be protein degradation products, perhaps of the histones. However, it has been shown that all four of the major HMG proteins are present in neutral 0.35M NaCl extracts of chromatin, indicating that they are not products of acid hydrolysis. Furthermore, the proteins can be prepared from tissues removed from the animal immediately after death and homogenized directly with 0.75M $HClO_4$, indicating that they do not arise in the preparative procedure through the activity of endogenous proteases (7).

The amino acid composition of protein 14 shows that it is a highly polar molecule being composed of 26% basic amino acids and 25% acidic amino acids. Indeed, the amino acid composition of the four HMG proteins, 1, 2, 14 and 17 are similar, all of them being dominated by five amino acids, aspartic acid,

glutamic acid, proline, alanine and lysine. Considering the amounts of these proteins present in calf thymus is approximately 3% by weight of the histones, it is possible that these proteins could be associated with specific regions of the genome. However, the above figure is an average value for the tissue and it may be that the HMG proteins are present and function only during a particular stage of the cell cycle.

ACKNOWLEDGEMENTS

I thank Dr M.J.P. Higgins for helpful discussions and Dr P. Lowry of St Bartholomew's Hospital, London, for generously doing the amino acid analyses.

REFERENCES

1. Johns, E.W., Goodwin, G.H., Walker, J.M. and Sanders, C. (1975) in: The Structure and Function of Chromatin, Ciba Foundation Symposium, No. 28, pp. 95-108, Associated Scientific Publishers, Amsterdam.
2. Goodwin, G.H., Sanders, C. and Johns, E.W. (1973) Eur. J. Biochem. 38, 14-19.
3. Goodwin, G.H., Nicolas, R.H. and Johns, E.W. (1975) Biochim. Biophys. Acta 405, 280-291.
4. Sanders, C. and Johns, E.W. (1974) Biochem. Soc. Trans. 2, 547-550.
5. Goodwin, G.H., Woodhead, J.L. and Johns, E.W. (1977) FEBS Letters 73, 85-88.
6. Goodwin, G.H. and Johns, E.W. (1973) Eur. J. Biochem. 40, 215-219.
7. Sanders, C. (1975) Ph.D. Thesis, London University.
8. Kirkegaard, L.H. (1973) Biochemistry 12, 3627-3632.
9. Goodwin, G.H., Nicolas, R.H. and Johns, E.W. (1976) FEBS Letters 64, 412-414.
10. Johns, E.W. (1969) J. Chromatogr. 42, 152-153.
11. Johns, E.W. (1967) Biochem. J. 104, 78-82.
12. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.