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Note

Purification of high mobility group nonhistone chromosomal proteins by liquid chromatography on a column containing immobilized histone H5

LISA WEN and GERALD R. REECK*
Department of Biochemistry, Kansas State University, Manhattan, KS 66506 (U.S.A.)
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The high mobility group (HMG) proteins are a small and intensively studied set of nonhistone chromosomal proteins (for a review see ref. 1). Besides interacting with DNA, at least some of the HMG proteins can bind to histones. HMG-1 and HMG-E (a member of the HMG-1 family) have been shown to interact with histone H1 in solution[2,3] and with histone H1 immobilized on agarose[4]. We have observed recently that each member of the chicken erythrocyte HMG-1 family (*i.e.* HMG-1, HMG-2 and HMG-E) binds to histone H1 or histone H5 and that these three HMG proteins have different affinities for those histones[5]. That observation suggests the possible usefulness of chromatography of HMG proteins on a column containing immobilized histone H1 or H5. We report that such chromatography on immobilized histone H5 is indeed a powerful fractionation method for chicken erythrocyte HMG proteins. It can provide not only good separation of nearly all the HMG proteins from each other but also partial separation of multiple forms of the proteins, most effectively of HMG-14.

MATERIALS AND METHODS

Preparation of HMG proteins

Chicken erythrocyte chromatin was prepared as previously described[6,7]. The chromatin was adjusted to 0.2 *M* sodium chloride by adding solid sodium chloride and subjected to centrifugation at 25,000 *g* for 30 min. The supernatant contained each of the HMG proteins (HMG-1, HMG-2, HMG-E, HMG-14, and HMG-17) but essentially no histone H1, as expected[8].

The 0.2 *M* sodium chloride extract was concentrated by adsorption to a Bio-Rex 70 column. Extract (500 ml) was applied to a 30 × 2.5 cm column that had been equilibrated to 0.2 *M* sodium chloride–50 m*M* Tris–HCl (pH 7.5). All of the HMG proteins bound. They were eluted in a concentrated peak by applying 1.0 *M* sodium chloride to the column. The concentrated sample was dialyzed against 10 m*M* Tris–HCl (pH 7.5). Some insoluble material that formed during dialysis was removed by centrifugation. The soluble material was the sample used for chromatography on a histone H5 column as described below.

A mixture of HMG-14 and HMG-17 was prepared for electrophoretic analysis

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by extracting chicken erythrocyte nuclei with 15% (w/v) trichloroacetic acid. The supernatant obtained after centrifugation at 10,000 *g* for 15 min contained HMG-14 and HMG-17. It was brought to 25% (w/v) trichloroacetic acid by adding the appropriate amount of 100% (w/v) trichloroacetic acid. The precipitate was collected by centrifugation, washed twice with acetone, and dissolved in the lysis buffer of O'Farrell *et al.*[9].

Preparation of histone H5

Histones H1 and H5 were extracted with 5% perchloric acid from chromatin that had been treated with 0.2 *M* or 0.35 *M* sodium chloride. H1 and H5 were purified from the extract using an approach described previously[5] in which chromatography on Bio-Rex 70 is the last step. Using a 25 × 3 cm column, we obtained about 300 mg of purified histone H5 from each chromatographic separation.

Construction and use of the histone H5 column

Purified histone H5 was coupled to cyanogen bromide-activated Sepharose 2B as previously described[10]. The resulting agarose (100 ml containing 270 mg of covalently coupled histone H5) was packed in a column (50 × 1.5 cm) and used to fractionate samples containing HMG proteins.

The mixture of HMG proteins obtained by extracting chromatin with 0.2 *M* sodium chloride was processed as described above and applied to the histone H5 column, which had been equilibrated to 10 m*M* Tris–HCl (pH 7.5). After the flow-through peak had emerged, the column was washed with 300 ml of 10 m*M* Tris–HCl (pH 7.5) to ensure removal of all unadsorbed material. A linear sodium chloride gradient from 0 to 1.0 *M* sodium chloride in a total volume of 800 ml of 10 m*M* Tris–HCl (pH 7.5) was applied to elute bound proteins. The chromatography was carried out at 4° C at a flow-rate of 100 ml/h.

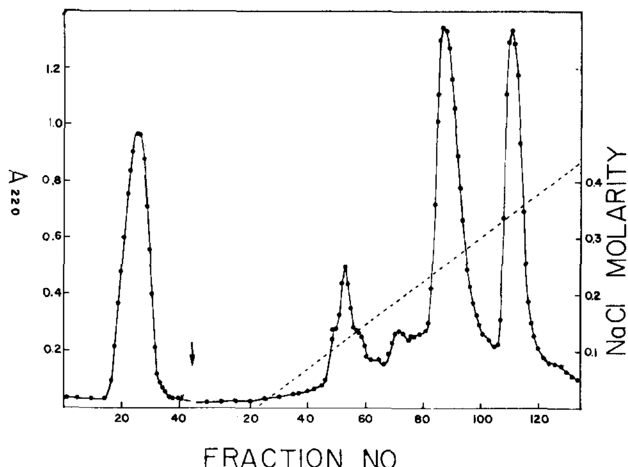
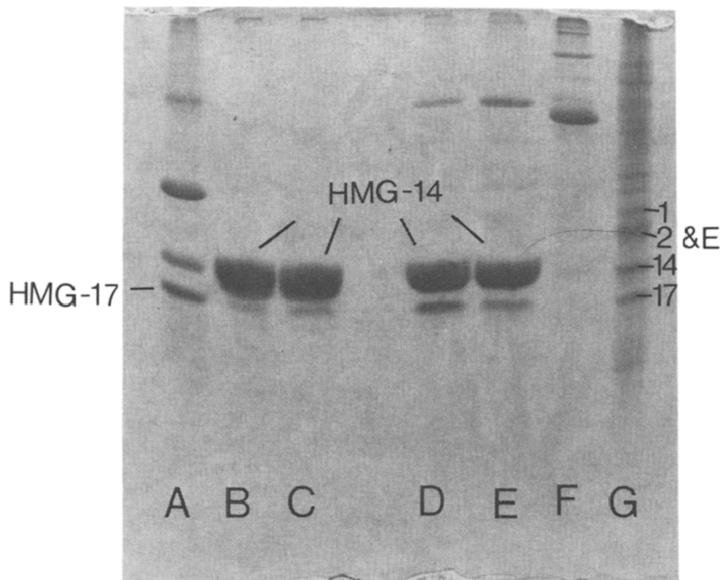


Fig. 1. Chromatography of chicken erythrocyte HMG proteins on histone H5-Sepharose. Approximately 70 mg of HMG proteins was applied to a 50 × 1.5 cm column which contained about 270 mg of H5. After the sample was applied, the column was washed with 10 m*M* Tris–HCl (pH 7.5). Bound proteins were then eluted with a linear salt gradient (starting at the arrow). Sodium chloride concentrations were determined by conductivity measurements of individual fractions. The fraction volume was 2 ml.



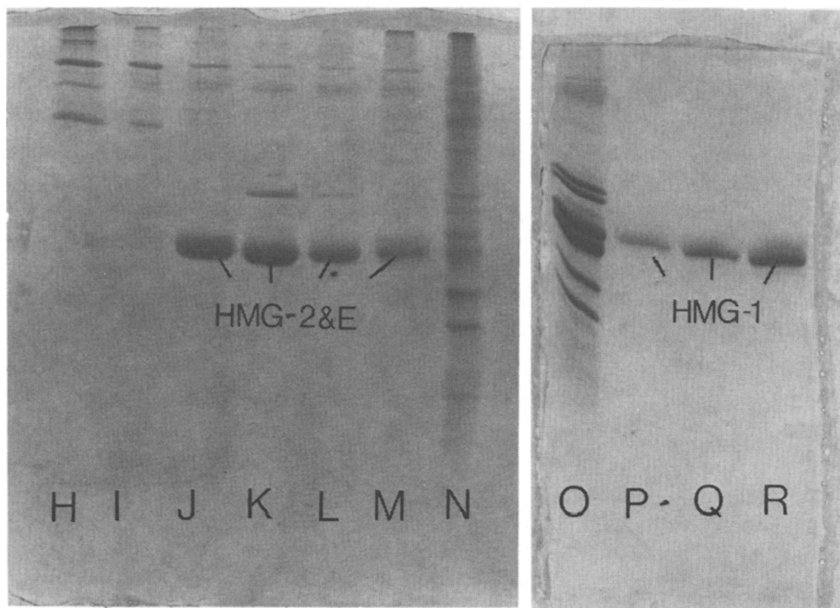


Fig. 2. SDS polyacrylamide gel electrophoresis of HMG proteins fractionated by chromatography on a histone H5 column. Samples for electrophoresis were as follows (fractions refer to the elution profile of Fig. 1): A, flowthrough (fractions 18–32); B, fraction 49; C, fraction 51; D, fraction 55; E, fraction 58; F, fraction 70; G, 0.35 *M* sodium chloride extract of chicken erythrocyte chromatin (for reference); H, fraction 74; I, fraction 80; J, fraction 85; K, fraction 90; L, fraction 95; M, fraction 101; N and O, 0.35 *M* sodium chloride extract of chicken erythrocyte chromatin (for reference); P, fraction 111; Q, fraction 114; R, fraction 117. The band in A with the mobility of HMG-14 does not stain with the characteristic purple color of HMG-14 and is therefore presumed not to be HMG-14.

Gel electrophoresis

Two-dimensional gel electrophoresis was performed as described previously[5] with non-equilibrium pH gradient electrophoresis in the first dimension and SDS–polyacrylamide gel electrophoresis in the second dimension. For optimal resolution of multiple forms of HMG-14, LKB ampholytes of pH range 9–11 were used in the first dimension.

RESULTS

Fig. 1 shows the elution profile resulting from chromatography of an HMG protein-containing sample on a column constructed with histone H5. SDS–polyacrylamide gel electrophoretic analysis of several fractions of the effluent is shown in Fig. 2. The flowthrough from the chromatography contained HMG-17 and other, unidentified proteins. The other HMG proteins were retained by the column and were eluted with a sodium chloride gradient. They are eluted in the order: HMG-14 (fractions 46–62), HMG-2 and HMG-E (fractions 84–100) and, lastly, HMG-1 (fractions 110–120).

Protein fractions also were analyzed by two-dimensional gel electrophoresis in

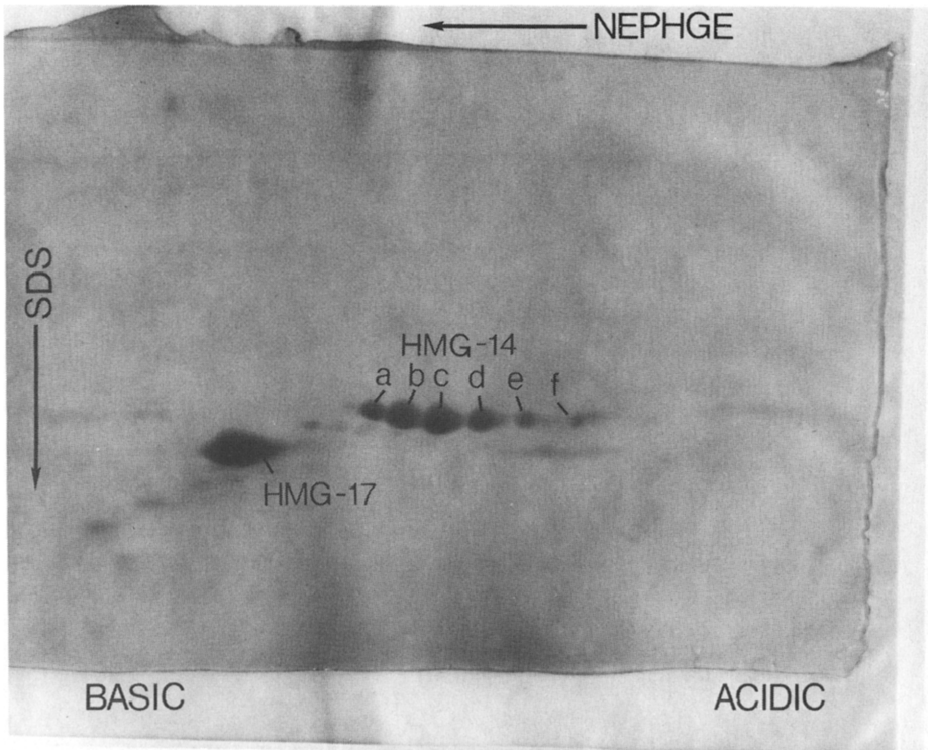


Fig. 3. Two-dimensional gel electrophoresis of chicken erythrocyte HMG-14 and HMG-17. The sample was prepared as described in Materials and Methods. The first-dimension gel contained pH 9–11 ampholines, and the electrophoresis was carried out for 2500 volt-hours. The forms of HMG-14 are labeled a through f for comparison with electrophoretograms of Figs. 4–6.

order to see if any of the multiple forms[5,7] of individual HMG proteins were resolved by the chromatography on immobilized histone H5. The best resolution of multiple forms was observed for HMG-14. Multiplicity within this protein is shown in the electrophoretogram in Fig. 3, for which the sample was a mixture of HMG-14 and HMG-17 (prepared for electrophoresis as described in Materials and Methods). The resolution of six forms of HMG-14, labeled a through f in Fig. 3, has resulted from continued refinement in our first dimension electrophoresis conditions. In our initial two-dimensional analysis[7], HMG-14 was simply a distorted spot on the electropherogram. At an intermediate stage[5] several HMG-14 spots were observed, but they were not resolved as clearly as those shown in Fig. 3.

Two-dimensional electrophoretic analysis (Fig. 4–6) of three portions of the HMG-14 peak from the chromatogram shown in Fig. 1 demonstrates that the histone H5 column is capable of partially resolving the multiple forms of HMG-14. The relationship of HMG-14 spots shown in Figs. 4–6 to the multiple forms of HMG-14 shown in Fig. 3 was established by electrophoresis (not shown) of mixtures of the sample for Fig. 3 with the individual samples for the electropherograms of Fig. 4–6. The shoulder on the leading edge of the HMG-14 peak in the elution profile (fraction 48 in Fig. 1) contains the HMG-14 species a and b (Fig. 4). The center of the HMG-14

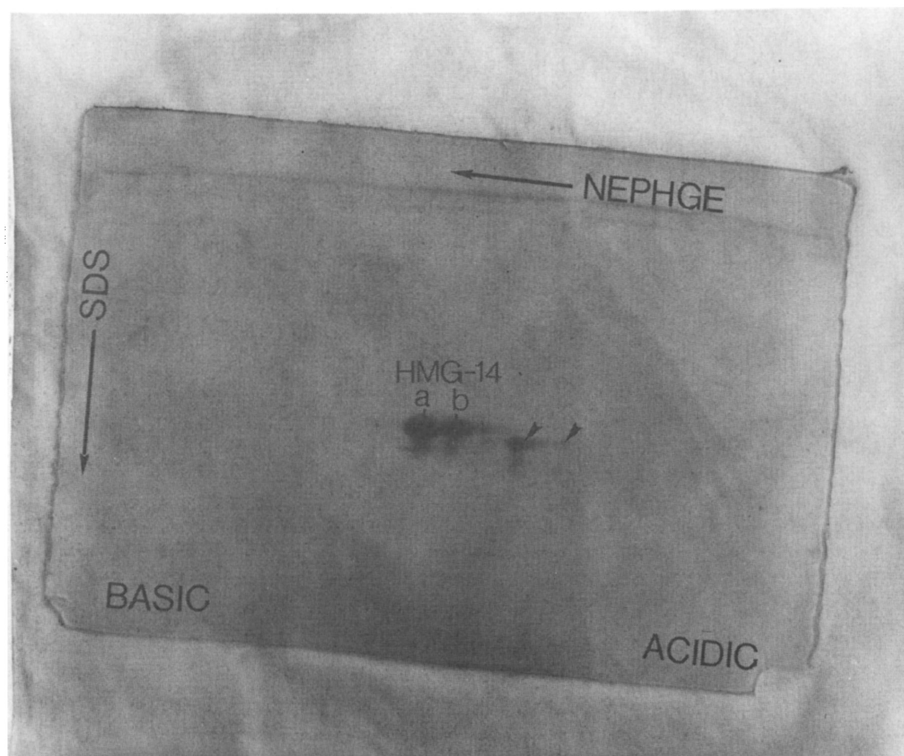


Fig. 4. Two-dimensional gel electrophoresis of fraction 48 from the chromatogram shown in Fig. 1. HMG-14 forms are labeled a and b, using the nomenclature of Fig. 3. The arrows indicate HMG 14/17-like proteins.

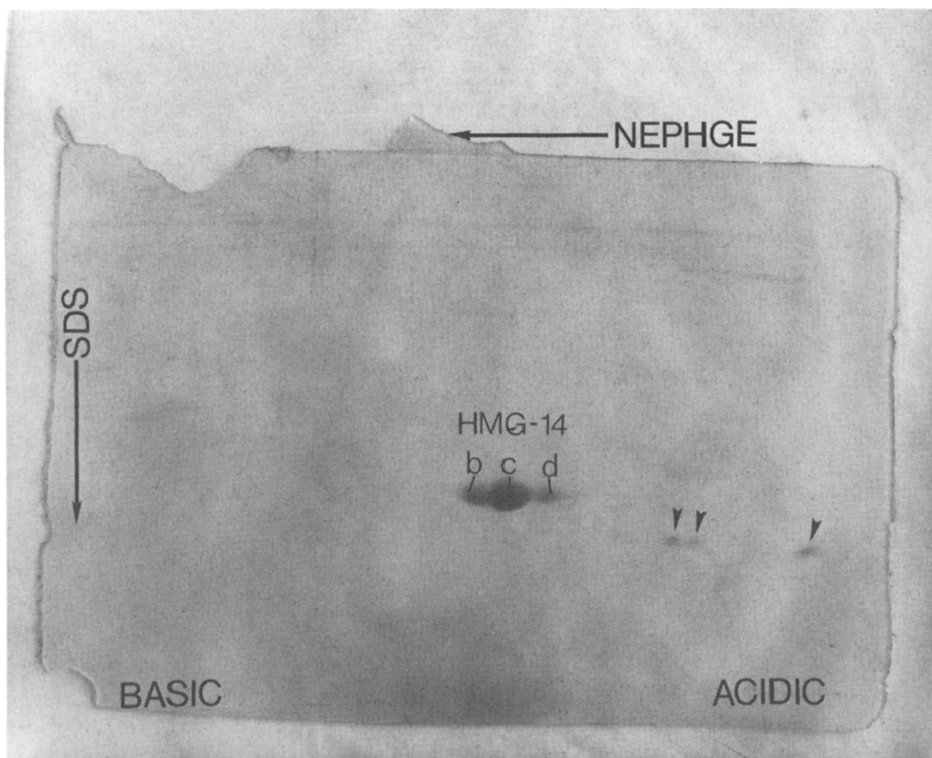


Fig. 5. Two-dimensional gel electrophoresis of fraction 55 from the chromatogram shown in Fig. 1. HMG-14 forms are labeled b through d, using the nomenclature of Fig. 3. The arrows indicate HMG 14/17-like proteins.

chromatographic peak (fraction 55) contains species b, c, and d (Fig. 5). The trailing edge of the HMG-14 peak (fraction 59) contains species c, d and e (Fig. 6).

Two-dimensional electrophoretic analysis (not shown) of other individual fractions of Fig. 1 demonstrated partial separation within the peaks of multiple forms of HMG-1, HMG-2 and HMG-E. In each case, the more basic a species of one of those proteins is, the earlier that species is eluted from the H5 column[11].

One final aspect of our results deserves mention. The HMG-14 peak contains polypeptides that appear to be related to HMG-14 and HMG-17 (see Figs. 4 and 5) but do not have precisely the electrophoretic properties that we take to be characteristic of those proteins. These polypeptides are indicated by arrow heads in Figs. 4 and 5. Two of the polypeptides (Fig. 4) have a slightly greater mobility than does HMG-14 in the second dimension. Another set of three polypeptides (Fig. 5) has a mobility in the second dimension quite similar to that of HMG-17. The three polypeptides are, however, much less basic than is HMG-17 and, unlike HMG-17, they are retained by the histone H5 column. These five spots (in Figs. 4 and 5) may well be related to HMG-14 and HMG-17 since they have the same Coomassie blue staining characteristics (a purple color) as do HMG-14 and HMG-17, and they also have the extraordinary solubility in trichloroacetic acid that is characteristic of HMG-14

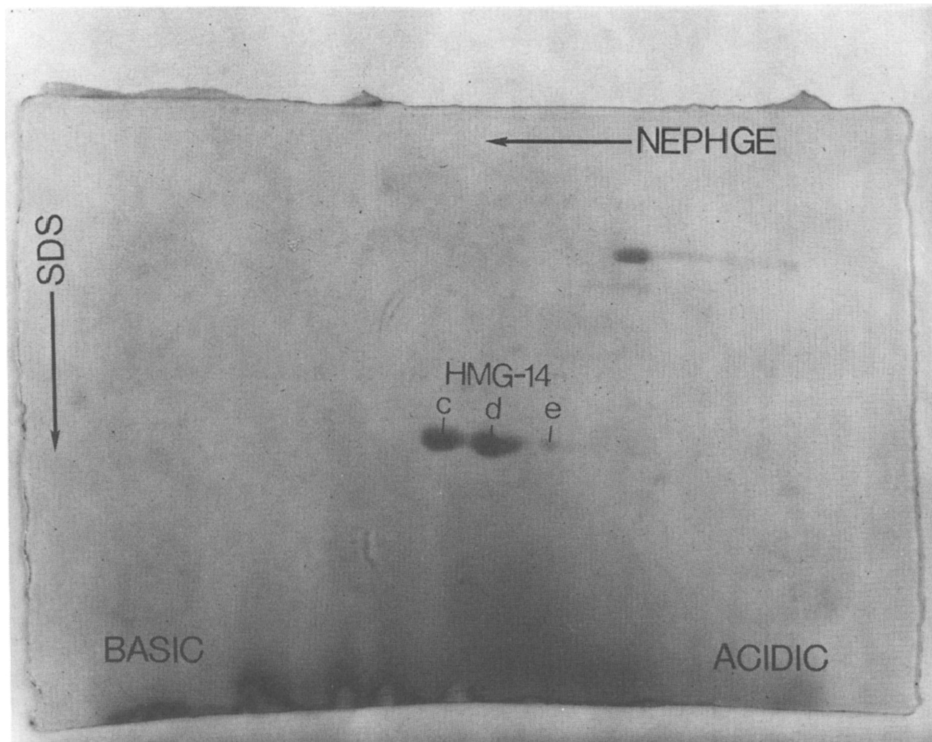


Fig. 6. Two-dimensional gel electrophoresis of fraction 59 from the chromatogram shown in Fig. 1. HMG-14 forms are labeled c through e, using the nomenclature of Fig. 3.

and HMG-17. (They can be seen in Fig. 3, the sample for which was prepared by trichloroactic acid extraction.)

DISCUSSION

Our results demonstrate that chromatography on a column containing immobilized histone H5 is a powerful fractionation method for chicken erythrocyte HMG proteins. Indeed, in the case of HMG-1 and HMG-14, preparations of high purity can be obtained in this single chromatographic step. We also have used the H5 column successfully to chromatograph HMG proteins from calf thymus, rat liver, and cultured mouse L cells (not shown). Thus, the method appears to be generally useful in purifying HMG proteins, especially the members of the HMG-1 family.

It is in combination with our previously described phosphocellulose chromatography of HMG proteins[6] that the H5 chromatography exhibits its most attractive feature. Because the order of elution of the individual HMG proteins is nearly exactly reversed in the two systems, contaminants remaining after chromatography on phosphocellulose can, in every case we have examined, be nicely removed by subsequent chromatography on histone H5. Because it is easier to prepare high-capacity phosphocellulose columns than high-capacity histone H5 columns, the or-

dinary sequence of the two systems for large scale purification would be chromatography on phosphocellulose followed by chromatography on histone H5.

At first glance, it is surprising that HMG proteins bind to histones since, with the exception of HMG-1, the HMG proteins are strongly basic proteins[5,7], as are the histones. The members of the HMG-1 family, however, have an extraordinarily acidic C terminal region[12] that we have termed domain C[13]. It is presumably through this region that HMG-1 binds to histones. In calf thymus, the C domain of HMG-1 is somewhat more acidic than is the C domain of HMG-2: those regions have net charges at neutral pH of -30 and -26, respectively[12]. This may explain why HMG-1 (from chicken erythrocytes in our case) binds more tightly to H5 than does HMG-2. HMG-14 also has an asymmetry in its charge distribution[14], and the last half of the molecule carries a net charge of -9. Such asymmetry is much less striking in HMG-17[15], and that is presumably responsible for the fact that HMG-17 does not bind to a histone H5 column.

Although the interaction of HMG proteins with histone H5 can be presumed to be largely, if not entirely, electrostatic, the quality of the separation we have obtained is dependent upon having histone H5 as the immobilized positive charges. Inferior separations were obtained by chromatography of HMG protein samples on immobilized histone H1 or on DEAE-cellulose[11].

It is not certain that the interactions of HMG proteins with histones are physiologically relevant. It is worth noting, however, that relatively high sodium chloride concentrations are required to dissociate certain HMG proteins, particularly HMG-1, from histone H5. Thus the HMG-1–H5 interaction appears to be strong enough to occur in living cells. Whether it does occur *in vivo* is another matter, of course. The molecular architecture of the members of the HMG-1 family does suggest, however, that these proteins are designed to interact simultaneously with DNA and with histones through physically distinct domains[1,13,16]. In that context, it is interesting to note that the relative affinities of the members of the HMG-1 family for DNA, on the one hand, and for histone H5, on the other hand, run in opposite directions through the family of proteins (as evidenced by the opposite orders of elution of the proteins from DNA columns[17] and from a histone H5 column). Furthermore, at least for the interaction with histone H5, there appears to be a range of affinities within the subspecies of each HMG protein (as evidenced by partial resolution of multiple forms by chromatography on immobilized histone H5). Thus, it appears that the HMG-1 family has a rather finely graded range of affinities from the most basic form of HMG-2 to the least basic form of HMG-1, with affinity for histones increasing and affinity for DNA decreasing in that sequence. The shifts observed in the relative amounts of members of the HMG-1 family[18,19] suggest that there is a physiological relevance to the differences in affinities, and that under certain (as yet obscure) circumstances, increases in the relative amounts of certain of the proteins are required or desirable.

ACKNOWLEDGEMENTS

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