Heterogeneity of proteins resembling high-mobility-group protein HMG-T in trout testes nuclei

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Two proteins, HMG-T1 and HMG-T2, with electrophoretic mobilities and compositions similar to those of protein HMG-T, were isolated from trout testes nuclei. The isoelectric points of proteins HMG-T1, HMG-T2 and HMG-T differ. The first 20 residues of protein HMG-T2 have been sequenced and differ from protein HMG-T by only one residue.

The HMG proteins are present in the somatic cell nuclei of many widely divergent eukaryotic species. They are present in about one-tenth of the amount of the predominant proteins of chromatin, the histones, which are basic, structural proteins. The HMG proteins appear to vary considerably more from species to species than do the four core histones, which have been highly conserved throughout evolution. The extent of their species and tissue specificity has been reviewed by Goodwin *et al.* (1978) and is currently being amplified by further reports from other laboratories (Hamana & Iwai, 1979; Teng *et al.*, 1979; Weber & Isenberg, 1980). In mammals and birds, two of the larger proteins, HMG1 and HMG2, form a very closely related pair. In fish, only one protein similar to the HMG1 and HMG2 pair has been described. This protein, HMG-T, has been isolated from trout testis (Watson *et al.*, 1977) and trout liver (Rabbani *et al.*, 1980). However, on SDS/polyacrylamide-gel electrophoresis, at least two bands are frequently seen running in the position of protein HMG-T, suggesting that there may be other closely related forms of this protein, as in more highly evolved species.

Experimental

Preparation of nuclei and extraction of protein

Trout testes (300 g), which had been stored in liquid N₂, were blended at low speed for 30 s in

2 litres of 0.25 M-sucrose/3 mM-MgCl₂/0.5 mM-phenylmethanesulphonyl fluoride, and centrifuged for 15 min at 2000 g. The pellet was resuspended in 1.5 litres of 1.8 M-sucrose/3 mM-MgCl₂/0.5 mM-phenylmethanesulphonyl fluoride, and centrifuged for 1 h at 30 000 g. The purified nuclei were washed with 0.25 M-sucrose/3 mM-MgCl₂/0.5 mM-phenylmethanesulphonyl fluoride, and recovered by centrifuging for 10 min at 2000 g. Electron micrographs of the final pellet showed that the nuclei were free of cytoplasmic contamination. Histone H1 and HMG proteins were extracted with HClO₄ as described by Sanders & Johns (1974), and precipitated together by acidification to 0.3 M with HCl, followed by the addition of 6 vol. of acetone.

Column chromatography

The HClO₄-extracted protein (histone H1 + HMG) was dissolved in 30 ml of 7.5 mM-sodium borate buffer, pH 9.0, and was dialysed overnight at 4°C against the same buffer containing 0.15% (v/v) mercaptoethanol. Before the sample was loaded, urea and buffer were added to give a final concentration of 9 M-urea and 0.05% (v/v) mercaptoethanol in a total volume of 90 ml. It was titrated slowly to pH 9.0 by the addition of 1 M-NaOH, centrifuged at 5000 g for 10 min and loaded on to a column (5 cm × 30 cm) of CM-cellulose equilibrated with 7.5 mM-sodium borate buffer, pH 9.0. Then 250 ml of buffer was pumped through the column at a flow rate of 0.8 ml/min, followed first by a 700 ml linear gradient of 0–0.3 M-NaCl in the buffer and then by a 1.2 litre linear gradient of 0.3–1.2 M-NaCl. Fractions (a)–(h) were pooled as

Abbreviations used: HMG protein, high-mobility-group protein; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; SDS, sodium dodecyl sulphate.

indicated in Fig. 1 and protein was recovered by acetone precipitation.

Analysis of proteins

Proteins were analysed by electrophoresis on 15% (w/v) polyacrylamide gels in 0.1% (w/v) SDS as described by Laemmli (1970) and on acid/urea gels as described by Panyim & Chalkley (1969). Isoelectric focusing was carried out by the method of Goodwin *et al.* (1976).

N-Terminal amino acids and amino acid composition were determined as described previously (Walker *et al.*, 1976). Sequence determinations were carried out on a Beckman 890C sequencer as described by Walker *et al.* (1980).

Results and discussion

The elution profile of the HClO_4 -extracted proteins from trout testes nuclei is shown in Fig. 1, and proteins recovered by acetone precipitation from fractions (a)–(h) and electrophoresed on a SDS/polyacrylamide gel are shown in the corresponding lanes in Fig. 2(a). Figs. 2(a) and 2(b) show that two proteins, later designated proteins HMG-T1 and HMG-T2, were eluted early, and have similar mobilities to those of HMG1 and HMG2. Watson *et al.* (1977) have already shown that protein HMG-T also has the same mobility as proteins HMG1 and HMG2 on SDS/polyacrylamide gels. It is noteworthy that proteins HMG-T1 and HMG-T2 are eluted early from the column, like calf thymus proteins HMG1 and HMG2. The earlier-eluted

protein of the two (HMG-T1) is seen most strongly in lanes (b), (c) and (d), and the second (HMG-T2), which migrates less rapidly, is seen strongly in lane (h) (Fig. 2a). Since proteins HMG1 and HMG2 are known to contain at least two residues of cysteine (Walker *et al.*, 1976) and to run on polyacrylamide gels as doublets when not fully reduced (Baker *et al.*, 1976), it was important to ascertain whether or not the protein eluted earlier from the column was merely an oxidized form of the protein in fraction (h). Samples from each fraction were therefore fully reduced by overnight incubation in mercaptoacetic acid. On an acid/urea gel, after such full reduction, each protein migrated as a single band, the major protein in fraction (h) migrating, at low pH, marginally faster than the earlier-eluted protein; on an SDS/polyacrylamide gel, the relative mobilities of the two proteins remained unchanged, the earlier-eluted protein running unmistakably more rapidly than the major protein in fraction (h), as in Fig. 2 (results not shown).

The proteins in fractions (c), (d) and (h) were sufficiently pure for amino acid analysis. The analyses of fractions (c) and (d) were almost identical. Since fraction (d) was known to be completely free of urea, this analysis was given in Table 1, together with the analysis of protein from peak (h), and of protein HMG-T taken from Watson *et al.* (1977). [The value for glycine in the amino acid analysis of protein HMG-T is now known to be

Fig. 1. Ion-exchange chromatography of HClO_4 -extracted HMG proteins from trout testes nuclei on a CM-cellulose column in 7.5 mM-sodium borate buffer, pH 9.0

Fractions (10 ml) were collected and pooled as indicated by each horizontal bar. —, A_{280} ; ----, NaCl gradient. Protein HMG-T1 is eluted mainly in fractions b, c and d, and protein HMG-T2 mainly in fraction h.

Fig. 2. SDS/polyacrylamide gel (a) of total HClO_4 -extracted HMG proteins from trout testis nuclei and of fractions obtained by chromatography of the total protein and (b) of calf thymus HMG protein and HClO_4 -extracted trout testis nuclear protein

P, total 5%- HClO_4 -extracted protein from trout testes nuclei; a–h, individual fractions obtained by chromatography of total 5%- HClO_4 -extracted protein from trout testes nuclei on CM-cellulose as indicated in Fig. 1; protein HMG-T1 appears mainly in lanes b, c and d, and protein HMG-T2 in lane h. C, standard of total HMG proteins (partially purified) from calf thymus.

Table 1. *Comparison of the amino acid content of proteins HMG-T1, HMG-T2 and HMG-T*

Amino acid analyses of proteins HMG-T1 (fraction d, Fig. 1), HMG-T2 (fraction h, Fig. 1) and HMG-T (Watson *et al.*, 1977) are shown. Abbreviation: n.d., not determined.

Amino acid	Content (mol%) in protein:		
	HMG-T1	HMG-T2	HMG-T
Asp	15.2	12.4	11.32
Thr	3.0	4.0	3.02
Ser	8.0	6.6	4.53
Glu	12.9	9.7	9.06
Pro	7.4	9.9	7.55
Gly	8.0	9.3	17.36
Ala	8.8	11.5	8.30
Val	3.8	3.6	3.77
Cys	n.d.	n.d.	0.75
Met	0.9	1.5	1.89
Ile	2.0	1.1	1.51
Leu	3.0	1.6	2.64
Tyr	2.2	1.7	2.26
Phe	3.2	3.5	3.40
Lys	14.2	16.8	15.47
His	1.5	1.7	1.51
Arg	5.7	5.0	5.28
Trp	n.d.	n.d.	0.38

lower than the one given in Table 1 (G. H. Dixon, personal communication)]. A comparison of the three analyses showed that, with the exception of serine and glycine, all three resembled each other closely, and we have called the earlier-eluted protein HMG-T1, and the protein in fraction (h), HMG-T2.

That proteins HMG-T1 and HMG-T2 were indeed different proteins was confirmed by *N*-terminal amino acid analyses, which showed that protein HMG-T1 had glycine and HMG-T2 had proline as *N*-terminal residue. Isoelectric focusing of the two proteins also confirmed the difference. Fig. 3 shows that protein HMG-T2, when loaded at the anode, gave one main band and several minor subsidiary bands (Fig. 3, lane b), whereas, when it was loaded at the cathode, the main band only appeared (Fig. 3, lane g). Protein HMG-T1 focused as two bands whether loaded at the cathode or at the anode (Fig. 3, lanes c and f). These two bands, however, both focused at a different, lower, pH than did the main band of protein HMG-T2. Thus protein HMG-T2 appeared to be a slightly more basic molecule than protein HMG-T1, so confirming the higher content of basic residues found in the amino acid analysis of protein HMG-T2, and higher acidic acid content of protein HMG-T1.

The *pI* values of proteins HMG-T1 and HMG-T2 differed, not only from each other, but also from the *pI* reported by Watson *et al.* (1977) for HMG-T,

Fig. 3. *Isoelectric focusing of proteins HMG-T1 and HMG-T2 from trout testes nuclei and HMG1 and HMG2 from calf thymus*

Isoelectric focusing was carried out on a LKB Multiphor apparatus on a gradient formed between pH 3.5 and 9.5. Tracks (a), (h), calf thymus HMG2; (b), (g), trout testis HMG-T2; (c), (f), trout testis HMG-T1; (d), (e), calf thymus HMG1. Samples (a)–(d) were loaded at the anode and samples (e)–(h) were loaded at the cathode.

which focuses between pH 6.1 and 6.3. Also Watson *et al.* (1977) showed that protein HMG-T was eluted from a CM-cellulose column, pH 9.0, at 0.35 M-LiCl, whereas in the chromatography carried out by us on a similar column, protein HMG-T1 was eluted at zero salt and HMG-T2 at about 0.13 M-NaCl. However, both proteins HMG-T and HMG-T2 had the same *N*-terminal residue, proline, so it was decided to sequence the first 20 amino acids of protein HMG-T2. The amino acid sequence determined was unequivocal and, with one exception, identical with that of protein HMG-T. The residue found at position 15 was tyrosine, as in calf thymus proteins HMG1 and HMG2, and not serine, as found in protein HMG-T by Watson *et al.* (1977). The sequence of proteins HMG-1 and HMG-2 has been compared with that of protein HMG-T by Goodwin *et al.* (1978). The presence of a tyrosine residue at position 15 means that, with the exception of a threonine residue at position 12 instead of methionine, and another basic residue, lysine, at position 9 instead of arginine, there is a block of 16 amino acids between positions 4 and 19 where the sequence of protein HMG-T2 is identical with that of protein HMG-2. Thus protein HMG-T2 appears to resemble protein HMG2 rather than protein HMG1, as the latter has a lysine residue at position 6 instead of the asparagine residue of proteins HMG-T2 and HMG2.

It would appear that proteins HMG-T2 and HMG-T are very closely related and are probably microheterogeneous forms of the same molecule, just as protein HMG2 has four subfractions focusing at different *pI* values (Fig. 3, lanes a and b). It is possible that protein HMG-T1, although it has a different *N*-terminal residue, may constitute yet

another subfraction of this protein, as subfractions of protein HMG2 have been found to have different *N*-terminal residues in chicken thymus and erythrocytes (Mathew *et al.*, 1979). Preliminary isoelectric-focusing results indicate that protein HMG-T1 itself may be microheterogeneous (E. Brown, unpublished work) and may explain why the protein is eluted in several peaks [(b), (c) and (e), Fig. 1].

The precise relationship between proteins HMG-T1, HMG-T2 and HMG-T in fish, and proteins HMG1 and HMG2 in mammals and birds, is still unclear, but it is evident that microheterogeneity is a characteristic of these proteins.

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