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High Mobility Group Proteins in Ram Spermatids

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The four major high mobility group proteins HMG 1, 2, 14 and 17, HMG 19B and histone H₁^o were identified in the ram testis by their extraction and solubility characteristics and by their electrophoretic mobilities. HMG 14 and 17 were isolated by chromatography and amino acid analysis revealed that they were similar to their calf thymus analogues. A protein, named 2R and co-extracted with HMG 14, was also purified and analysed. Electrophoretic analyses of the proteins extracted by 0.75 M perchloric acid (PCA) or by 0.35 M NaCl from round and non-round spermatids, separated by centrifugal elutriation, showed that the four major HMG proteins disappear from nuclei in the oldest round spermatids, at the time the nuclear content of protein 2R and histone H₁^o increases in spermatids. Ubiquitin and HMG 19B were present in the round and elongating spermatids, but not in elongated spermatids which contained only protamine. The relation was considered between several protein changes and genetic inactivation and structural reorganization of the spermatid chromatin. © 1986 Academic Press, Inc.

In mammals, spermiogenesis provides a unique system for the study of cell differentiation in higher animals. In the early spermatids, the organization of chromatin is similar to that in somatic cells and transcription is very active [1–3]. Differentiation of the spermatids is accompanied by complete genome inactivation [1, 2, 4], progressive DNA packaging [5] and the replacement of histones, firstly, by several spermatidal proteins [6, 7] and ultimately, by one or more protamines (for review, see [8]).

In mammalian cells, the HMG proteins consist of four major species, HMG 1, 2, 14 and 17 and of a number of minor species [9, 10]. Transcriptionally active portions of the genome are enriched in the major HMG proteins, particularly HMG 14 and 17 which, however, are not entirely confined to actively transcribing chromatin [11]. Because of the original features of mammalian spermiogenesis, the biological function of the HMG proteins in spermatids was studied.

This paper deals with the identification of HMG proteins present in the ram testis and with their distribution in spermatid populations separated by centrifugal elutriation.

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MATERIALS AND METHODS

Testes of sexually mature rams were used either immediately after castration or after quick freezing in liquid nitrogen and storage at -20°C . No significant differences were observed between fresh and frozen testes. All procedures for the extraction of the testicular proteins were performed at 2°C .

Chromatin Preparation

Ram testis chromatin and calf thymus chromatin were prepared according to the method of Marushige & Marushige [12], in the presence of 0.1 mM phenylmethylsulfonyl fluoride (PMSF) throughout all steps of the process and of 50 mM sodium bisulfite during the homogenization step. Triton X-100 was used at a concentration of 0.1% instead of 0.5%, as described in [12].

Separation of Ram Spermatids and Nuclear Isolation

Three germ cell populations were separated by centrifugal elutriation as described previously [13]. The first population contained 96–100% elongated spermatids (maturation steps 13–15; ref. [13]). The second population ('non-round spermatid population') contained mostly (74–87%) elongating spermatids (steps 9–12) and was contaminated by a few elongated and round spermatids. The third population ('round spermatid population') contained 90–97% round spermatids (steps 1–8) and some spermatocytes, spermatogonia and non-round spermatids.

Nuclei were isolated from the different cell populations with 0.09% Triton X-100 in buffer A (5 mM Tris-HCl, pH 7.4, 3 mM CaCl_2 , 1 mM MgCl_2 , 1 mM PMSF). The nuclear suspension was diluted with 0.5 vol of 1.5 M sucrose in buffer A and centrifuged at 1 000 g for 30 min on 1.5 vol of 1.5 M sucrose in buffer A. The nuclear pellet was washed twice with buffer A.

The nuclear composition and nuclear concentration were determined for each population [7]. These two parameters were used to calculate the number of 'haploid genomes' for each population (1 spermatid=1 haploid genome; 1 spermatogonium or secondary spermatocyte=2; 1 primary spermatocyte=4).

Protein Extraction

HMG proteins were extracted from chromatin preparations, germinal cells and nuclei with 0.75 M perchloric acid (PCA) [14] or by dialysing the acid extract (two extractions with 0.2 M H_2SO_4) against 0.75 M PCA and removing the non-HMG proteins by centrifugation. HMG proteins were also extracted from spermatid nuclei with 0.35 M NaCl [15], the low mobility group proteins being removed by precipitation with 3% trichloroacetic acid (TCA) [16].

Extraction and Purification of Testicular Proteins HMG17 and HMG14

Protein HMG 17 was isolated by a modification of the method of Bhullar & Candido [17] (fig. 1). A total of 800 g of testis was extracted twice with 800 ml 0.2 M H_2SO_4 . After centrifugation (10 000 g, 1 h), the combined supernatants were dialysed against 0.01 M HCl. Solid Tris was added to adjust the pH to 7.0 and 20 mM 2-mercaptoethanol and 0.1 mM PMSF were added. TCA was added to 2% final concentration and solid ammonium sulfate to 15% saturation (at 2°C). The solution was clarified by two successive centrifugations (12 000 g, 1 h) and concentrated to 650 ml in a hollowfibre Amicon concentration system (membrane cartridge type H_1P_2). The ammonium sulfate concentration was then increased stepwise first to 70% and secondly to 86% saturation. The proteins precipitated at 85% saturation were collected by centrifugation (12 000 g, 2 h), then dissolved in 40 ml 0.2 M H_2SO_4 and dialysed against 0.01 M HCl. After centrifugation at 18 000 g for 30 min, they were lyophilized. HMG 17 was purified by cation exchange chromatography on a CM-Sephadex C25 column (20×1.6 cm) eluted with a linear gradient of NaCl (from 0.25 to 0.50 M) in 15 mM sodium borate, pH 8.5. The yield of HMG 17 was 8.5 mg.

Protein HMG 14 was isolated in a similar way (fig. 1). A total of 500 g of testis was homogenized in 1 litre of 0.3 M H_2SO_4 . After centrifugation (10 000 g, 1 h), the pellet was extracted once with 400 ml 0.2 M H_2SO_4 . After one further centrifugation, the two supernatants were pooled and 100% TCA was added, with vigorous stirring, up to 7% final concentration. After centrifugation (18 000 g, 30 min) solid ammonium sulfate was slowly added to the supernatant, up to 65% saturation (at 2°C) and stirring was continued for 2 h. The precipitate was collected by centrifugation then dissolved in 60 ml

Fig. 1. Scheme of preparation of ram testicular proteins HMG 14, HMG 17 and 2R.

0.2 M H₂SO₄ and dialysed against 0.01 M HCl before being lyophilized. Approx. 100 mg of dry protein were obtained and dissolved in 14 ml of a solution of 8 M urea, 0.5 M 2-mercaptoethanol in 0.01 M HCl, 0.15 M NaCl. After dialysis for 3 h against 0.01 M HCl, 0.15 M NaCl, the protein solution was applied to a BioGel P10 column (100×3.2 cm) eluted with 0.01 M HCl, 0.15 M NaCl. The fractions of interest were combined, dialysed against 0.01 M HCl, and lyophilized. The proteins thus obtained were dissolved in 20 ml 0.2 M H₂SO₄ and dialyzed against 0.1 M NaCl, 15 mM sodium borate, 1 mM PMSF, pH 8.5. The solution was applied to a CM-Sephadex C25 column (25×1.6 cm) eluted as above except that the gradient varied from 0.18 to 0.50 M NaCl. The fractions containing the protein HMG 14 were pooled and dialysed against 0.01 M HCl and lyophilized.

Analytical Gel Electrophoresis

Protein samples were routinely analysed by electrophoresis on slab gels containing 2.5 M urea [18]. The gels were stained with 0.25 % Coomassie Brilliant Blue R250 [19] and scanned in a Vernon PHI 5 photometer. SDS-polyacrylamide gels were run according to the method of Laemmli [20]. For two-dimensional analysis, the method developed by Orrick et al. [19] and modified by Goldknopf & Busch [21] was used.

Amino acid analyses

Protein samples were hydrolysed in sealed evacuated tubes at 110°C for 24 and 72 h in 6 M HCl (1 ml/mg of protein). One drop of 1 % phenol was added to prevent excessive degradation of tyrosine.

Fig. 2. Electrophoretic analyses on acetic acid-urea gels of 0.75 M PCA-soluble proteins from (A) calf thymus chromatin; (B) ram testis chromatin; (C) directly extracted from ram testis; (D) proteins present in the first supernatant after homogenization when preparing chromatin.

Amino acid analyses were performed by the single-column method on a Beckman model 119 CL amino acid analyser.

RESULTS

Characterization of Testicular HMG Proteins

The electrophoretic profiles of the 0.75 M PCA extracts from ram testicular chromatin (figs 2B, 3) ram testis (fig. 2C), round spermatids (fig. 5A) and round spermatid nuclei (fig. 5C) were very similar to that of the 0.75 M PCA extract from calf thymus chromatin (fig. 2A) with, however, the following differences: two spermatid-specific proteins (7 and T, ref. [7]) were present in ram testicular and chromatin extracts and histone H_1° was absent from round spermatids.

The major HMG proteins HMG 1 and HMG 2 and also histone H_1° were not purified from ram testis but they were identified in the various protein extracts by their solubilities in 0.75 M PCA and in 0.35 M NaCl and by their electrophoretic mobilities on the three different polyacrylamide gels. One basic protein previously named 12 [22, 23], whose molecular weight is 7900 [22], was identified in a similar way as being the protein HMG 19B [10]. Purification and characterization of ram testicular ubiquitin has been reported elsewhere [24]. Although present in testis (fig. 2C), ubiquitin was not detected in ram testicular chromatin (fig. 2B) or in spermatid nuclei (fig. 5C, D). It was found, however, in the first supernatant discarded after homogenization of testes when preparing chromatin according to [12] (fig. 2D).

The fractionation of the testicular acid-soluble proteins by 2% TCA and

Table 1. Amino acid composition of the ram testicular proteins HMG 14, HMG 17 and 2R

	HMG 17		HMG 14			Protein 2 R
	Ram testis	Calf thymus	Ram testis 2T ₁	Ram testis 2T ₂	Calf thymus	Ram testis
Asp	12.3	12.0	8.5	8.3	8.3	3.2
Thr	1.2	1.2	3.9	4.3	4.1	5.9
Ser	2.4	2.3	6.6	7.8	8.0	11.6
Glu	10.5	10.5	14.8	16.8	17.5	18.8
Pro	12.8	12.9	11.0	10.4	8.1	13.0
Gly	11.1	11.2	9.3	8.1	6.4	9.5
Ala	17.8	18.4	15.9	15.2	14.8	5.8
Cys	—	—	—	—	—	—
Val	2.3	2.0	3.6	3.6	4.0	1.4
Met	—	—	—	—	—	—
Ile	—	—	0.3	—	—	0.9
Leu	1.2	1.0	2.1	1.8	2.0	2.1
Tyr	—	—	—	—	—	—
Phe	—	—	—	—	—	—
His	—	—	—	—	—	—
Lys	23.5	24.3	20.2	21.6	21.1	17.3
Arg	4.6	4.1	3.8	5.2	5.4	10.4

The results, obtained from duplicate analyses of 24 and 72 h hydrolysates (fraction 2T₁; only 24 h hydrolysis) are expressed in moles/100 moles of amino acids recovered. Values for threonine and serine were obtained by linear extrapolations to zero hydrolysis time. Amino acid composition of calf thymus HMG 14 and HMG 17 [30] are given for comparison.

ammonium sulfate (fig. 1) yielded essentially the HMG 17 protein (previously named protein 5, refs [7, 22]). It was further purified by chromatography on CM-Sephadex C25. Its amino acid composition is presented in table 1 and is very similar to that of calf thymus HMG 17.

The ram protein band (previously named 2 in refs [7 and 22]) which exhibits the same electrophoretic mobility as calf HMG 14 (fig. 2) appeared to be heterogeneous on both acetic acid-urea gels and on two-dimensional gels (figs 2, 3). It was resolved into two components by BioGel P10 chromatography (fig. 4A). These two components named 2T and 2R were further submitted to ion exchange chromatography on CM-Sephadex C25 (fig. 4B). The component 2T was further resolved into two subfractions, 2T₁ and 2T₂, whose amino acid compositions are given in table 1 with that of calf thymus HMG 14. It appears that both 2T₁ and 2T₂ are typical HMG 14 proteins. Fraction 2T₁ might correspond to chemically modified HMG 14.

The amino acid composition of protein 2R appears in table 1, and is characterized by large amounts of glutamic acid, lysine, proline, serine and arginine. The absence of aromatic residues and the size of this protein (MW 12 000; ref. [22])

Fig. 3. Two-dimensional PAGE of the 0.75 M PCA-soluble proteins from ram testis chromatin.

argues strongly against the possibility that protein 2R results from proteolytic degradation of HMG 1 or HMG 2 proteins. The solubilities of protein 2R in 0.75 M PCA, 0.35 M NaCl and in 10% TCA are identical with those of HMG 14.

Protein Changes in Ram Spermatids

The proteins extracted with 0.75 M PCA from cells or purified nuclei from round spermatid and non-round spermatid populations were analysed on polyacrylamide gels (fig. 5A–D). In round spermatids, the four major HMG proteins and HMG 19B were present in both cellular and nuclear PCA extracts. The non-round spermatids (fig. 5B, D) were mainly characterized by the disappearance of the four major HMG proteins including HMG 14. Furthermore, chromatographic analysis on CM-Sephadex C25 of the proteins extracted from spermatid nuclei by 0.75 M PCA confirmed that only the protein 2R was present in non-round spermatid nuclei, while HMG 14 was undetected (data not shown). Protein 2R was also detected to a lesser extent in round spermatid nuclei.

HMG 19B was present in both round and non-round spermatids and its amount per haploid genome was not conspicuously different in the two cell populations. Two spermatid-specific proteins, 7 and T which are 0.75 M PCA-soluble [25] along with histone H₁^o were also present in the cellular and nuclear extracts from non-round spermatids. Ubiquitin was found only in cells (fig. 5A, B) but, notably, was also present in the cytoplasmic fractions obtained after lysis of the different spermatids to prepare the nuclei (data not shown). There was roughly the same amount of ubiquitin per haploid genome in both round and non-round spermatids.

The loss of the four major HMG proteins and the increase in the amount of protein 2R during the transition from round to non-round spermatids were also assessed by the electrophoretic analysis of the 0.35 M NaCl nuclear extracts (fig.

Fig. 4. Purification of ram testicular HMG 14. (A) Fractionation of 7% TCA-soluble ram testicular proteins on (left) BioGel P10; (right) electrophoretic profiles of fractions 1 and 2. (B) Ion-exchange chromatography of fraction A1, on (left) CM-Sephadex C25; (right) electrophoretic profiles of fractions 1-3.

5E,F). It must be emphasized that among the non-round spermatid nuclear proteins, protein 2R was preferentially extracted with 0.35 M NaCl. Ubiquitin was not detected in the 0.35 M NaCl nuclear extracts.

In agreement with previous results [7], no protein was extracted from the elongated (steps 13-15) spermatid nuclei.

Fig. 5. Analysis of the proteins extracted by 0.75 M PCA and 0.35 M NaCl from two populations of ram germ cells separated by centrifugal elutriation. (A, C, E) Round; (B, D, F) non-round spermatid population. (A, B) Cellular proteins extracted by 0.75 M PCA; (C, D) nuclear proteins extracted by 0.75 M PCA; (E, F) nuclear proteins extracted by 0.35 M NaCl and soluble in 3% TCA. The six densitometric scans were reported to 1×10^8 haploid genomes.

DISCUSSION

Our results show that the four major HMG proteins, the protein HMG 19B and ubiquitin are present in ram testis. These proteins have also been identified in the rat testis [23, 26]. In both species, the major HMG proteins are present in the round spermatid nuclei but they are undetected in the non-round spermatids.

Although no precise quantitative evaluation has been carried out, it is apparent that in ram germinal cells, the bulk of the proteins HMG 1, 2, 14 and 17 is bound to the nuclei. On the contrary, ubiquitin was not detected in either ram testicular chromatin or in purified ram germinal cell nuclei. However, the possibility cannot be excluded that this absence of ubiquitin was due to leakage when chromatin and nuclei were prepared. Other studies have also failed to detect ubiquitin in purified nuclei [10, 16, 26].

Since 1975, a series of publications strongly supports the notion that transcriptionally active portions of the genome are enriched in HMG proteins, although these proteins are also present in non-transcribed regions [11]. How these proteins participate in the control of gene expression is still not clearly understood. The absence of proteins HMG 1, 2, 14, and 17 in the non-round spermatid nuclei and their conspicuous presence in round spermatid nuclei indicate that these proteins leave the chromatin of the least round spermatids. It is at this time that the transcriptional activity shuts down, the genome being thus completely inactivated [1] in the non-round spermatids. Therefore, our results confirm that, in the ram as in the rat [26], the four major HMG proteins are associated with functional, nucleosomal [3] chromatin. In the majority of ram round spermatids, the ultrastructure of chromatin is interphase-like. Its structural reorganization starts only in the oldest round spermatids [5]. It has been proposed [5] that the first structural nuclear changes could be due to the removal of histone H₁. However, histone H₁, along with the other histones, just begins to leave chromatin in the oldest round spermatids, so that the first structural changes would more likely be related to the removal of the major HMG proteins. Such a hypothesis would fit the function proposed for the HMG proteins that they maintain the chromatin in a configuration necessary for its activity [11]. However, the tentative interpretation of the first structural nuclear changes by the removal of the HMG proteins is somewhat restricted by the simultaneous appearance of five other proteins which are involved either in spermatid chromatin condensation (proteins T, 7, 3, and P₁, refs [5, 27]) or during the differentiation process in somatic cells (histone H₁^o, ref. [28]).

Because of the conspicuous increase of protein 2 R in non-round spermatids, it cannot be excluded, in absence of additional data, that this protein could also participate in the first structural changes. It is noteworthy that the protein 2 R displays all the characteristics of the HMG proteins [9]. In addition, it is deficient in aromatic amino acids as is HMG 14 and has a similar molecular weight. However it differs from the HMG proteins presently known in vertebrates in that it has quite a high content in serine, a rather low content in alanine and a low

lysine/arginine ratio. Most of the ram [25] and rat [6, 29] spermatid specific proteins are also characterized, as compared with the somatic histones, by high contents in serine, low contents in alanine and an increase in arginine concomitant with a decrease in lysine, this last characteristic being still more evident in the subsequently appearing protamines. So, the knowledge of the sequence of protein 2R would be useful to elucidate if it should not be a spermatid-specific variant of a somatic type protein HMG 14.

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