TIGHTLY BOUND SOMATIC HISTONES IN MATURE RAM SPERM NUCLEI

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1. Introduction

The idea that histones play not only a structural role but may be also involved in the control of cell differentiation [1] meets a serious difficulty in the largely accepted view that in some species histones are completely lost and replaced by protamines during spermiogenesis. Thus, in mammals the only well-documented proteins in mature sperm nuclei are protamines rich in sulfhydryl groups. However, recent data [2,3] have revealed, besides protamines, the presence of tightly bound proteins in mature sperm nuclei of mammals, part of which could be extracted only in the presence of detergents. The other part was released only after the complete digestion of DNA with DNase I and contained proteins with an electrophoretic mobility corresponding to that of the nucleosomal somatic histones [2]. In addition, our electron microscopic studies on decondensed mature ram sperm nuclei revealed some DNA fibers with nucleosome-like beads resistant to treatment with guanidine chloride or with high salt and urea concentrations [4].

These data suggest that in the course of spermiogenesis somatic histones might have been preserved at some sites of the genome in a state which prevents their extraction. To investigate this possibility it was necessary to study the nature of the histone-like proteins of mature ram sperm nuclei and especially in the fraction of the tightly bound proteins.

Here, we show that histone antigenic determinants are present in mature ram sperm nuclei both in the fraction extractable with high concentrations of salt and urea and in the material resistant to this treatment.

2. Materials and methods

2.1. *Preparation and extraction of mature ram sperm nuclei*

Mature ram sperm nuclei free of possible contamination with somatic or spermatid nuclei were obtained by sonication of a cell suspension of ram ejaculates and subsequent treatment of the isolated heads with 1% SDS for 2 h at 25°C [2]. This procedure preserves only nuclei of mature mammalian spermatozoa [5]. A careful examination of such nuclei by phase contrast microscopy did not reveal even a single contaminating nucleus when 10^3 nuclei were examined. Neither did electron microscopic observations show the presence of somatic nuclear material.

The preparation of the different fractions was according to the scheme in [2]. Briefly, the mature SDS-resistant nuclei were decondensed in 0.25 M 2-mercaptoethanol, 2 M NaCl, 6 M urea and centrifuged at 190 000 × *g* for 18 h at 5°C to obtain a supernatant S_1 and a pellet P_1. The latter was reextracted by the same procedure which gave the pellet P_1' and a second supernatant S_1' containing only 5–10% of the protein content of S_1. The pellet P_1' was dispersed in 2% SDS and after 2 h at 25°C was fractionated by centrifugation into a supernatant S_2 and a pellet P_2. Finally P_2 was digested with DNase I and the insoluble material P_3 was collected by low-speed centrifugation.

2.2. *Isolation and fractionation of chromosomal proteins*

Purified nucleosomal histones from calf and sheep livers were extracted from isolated nuclei [6] or from chromatin [7], respectively. Calf and sheep histones, fraction P_3 of ram sperm nuclei and total chromosomal proteins of sheep liver chromatin were fractionated in 15% polyacrylamide–SDS gels [8].

2.3. *Preparation of antisera*

To obtain a standard antiserum against mammalian nucleosomal histones, rabbits were immunized with a complex of calf nucleosomal histones and yeast RNA

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[9]. The rabbits were bled after 48 h fasting. Antisera to rabbit IgG were obtained from a sheep immunized with chromatographically purified rabbit IgG.

Lipoproteins from the sheep antiserum were removed by the addition of 10% dextran sulfate and 1 M $MgCl_2$ to final conc. 0.5% and 0.16 M, respectively. The resulting precipitate was removed by centrifugation at 10 000 × *g* for 20 min at 2°C. The supernatant was dialyzed against 0.15 M NaCl followed by dialysis against 0.08 M $BaCl_2$ in 0.17 M NaCl to precipitate the remaining dextran sulfate. The resulting lipoprotein-free supernatant was dialyzed against saline in 0.015 M phosphate buffer (pH 7.4) [10].

2.4. *Purification of rabbit IgG*

Rabbit sera (20 ml) (preimmune and after immunization) was dialyzed against 0.015 M phosphate buffer (pH 8.0) and fractionated by chromatography on a DEAE-cellulose (Whatman DE-52) column (2 × 40 cm) washed and equilibrated with the same buffer. Elution was performed with a 0.015–0.30 M linear gradient of phosphate buffer and 5 ml fractions were collected [10]. Every second fraction (fig.1) was tested for histone antibodies by the microcomplement fixation reaction. Non-specific precipitation reaction of the histones with protein fractions from the column was checked by measuring the turbidity at 550 nm after addition of 0.1 mg histones to 0.2 ml tested fraction.

2.5. *Purification of sheep antibodies to rabbit IgG*

Chromatographically purified preimmune rabbit IgG (6 mg) (fractions 12–25, fig.1) were covalently linked to 5 g cyanogen bromide-activated Sepharose 4B (Pharmacia) [11] and 10 ml sheep antiserum were applied to a 0.5 × 5 cm column of this Sepharose. Non-specifically bound proteins were removed by successive washings of the column with saline-phosphate buffer (pH 7.4) followed by borate buffer (1 M NaCl, 0.1 M boric acid, 0.025 M $Na_2B_4H_7$, pH 8.5) containing 0.1% Tween 20. The sheep anti-rabbit IgG was eluted with 1 M acetic acid, immediately neutralized with 0.3 M phosphate buffer (pH 8.0) and dialyzed against saline–phosphate buffer (pH 7.4).

The protein content of purified IgG was estimated by measuring the absorption at 280 nm accepting a value of 1.4 for 1 mg/ml [11].

2.6. *Indirect immunofluorescence staining of histones [12]*

Purified sheep anti-rabbit IgG were labelled with fluoresceine isothiocyanate (FITC) [13]. The binding of the antibodies was performed either directly in the electrophoresis gels or after blotting the proteins on nitrocellulose paper. In the first case the gels were cut into strips and fixed for 2 h in 50% trichloroacetic acid. They were washed in Dulbecco's PBS to neutral pH and incubated for 24 h in anti-histone rabbit IgG (fraction 12, fig.1). After a 2 day washing with several changes of PBS the gels were washed for 1 h with 0.05% Tween 20 in PBS and then for 2 h with PBS. The gels were further incubated for 24 h with FITC-conjugated sheep anti-rabbit IgG and successively washed with 0.05% Tween in PBS and finally with PBS alone.

Blotting was performed on nitrocellulose paper (Schleicher and Schüll, BA 85, 0.45 μm). To that end the polyacrylamide–SDS gels were washed overnight in 0.35 M Tris–HCl (pH 8.8) and the proteins transferred to the paper electrophoretically as in [14] in the original electrophoresis buffer 4 times diluted and containing 20% methanol. After 2.5 h at 80 V and 150 mA the paper strips were soaked in 3% bovine serum albumin (BSA, Sigma, fraction 5) in PBS buffer at 40°C for 60 min. The paper strips were then washed in PBS buffer for 30 min and incubated in the anti-histone IgG (fraction 18 in fig.1, diluted 10 times with 3% BSA in PBS) for 90 min at 37°C. After another washing in PBS for 30 min the strips were incubated with the FITC-labelled sheep anti-rabbit IgG for 90 min at 37°C and washed again in PBS. The transfer was checked by staining the gel after blotting with Coomassie brilliant blue R-250 and by staining some strips with amidoblack before soaking in BSA.

Photographs were taken in UV light at 254 nm.

2.7 *Microcomplement fixation (MCF) reaction*

This was done as in [13] with a purified rabbit anti-histone IgG (fractions 13 and 14, fig.1). In all immunochemical tests preimmune IgG were used as controls.

Protein content was determined as in [15].

3. Results and discussion

To investigate the nature of the histone-like proteins in the sperm nuclear fractions S_1, S_2 and P_3, we obtained antisera against calf liver nucleosomal histones (CLH). The antisera were purified on DEAE-cellulose and the most active IgG fractions (no. 12–18,

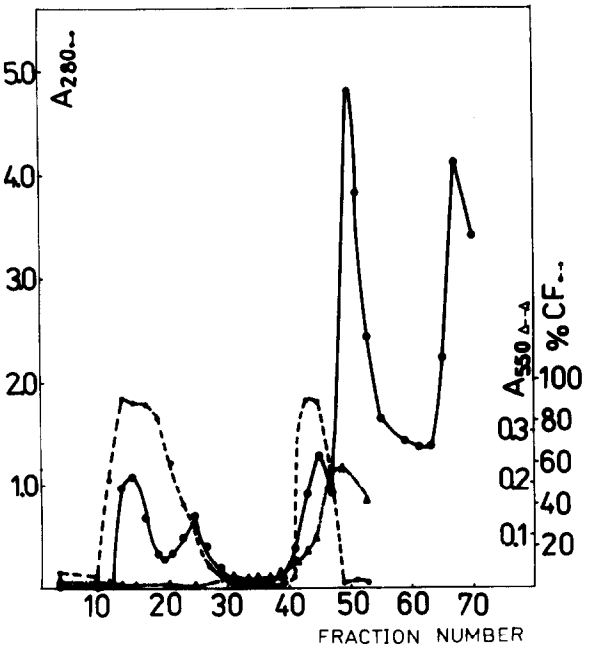

Fig.1. Purification of rabbit IgG on a DEAE-cellulose column: (●——●) absorption at 280 nm; (Δ——Δ) turbidity at 550 nm upon addition of histones; (○——○) complement fixation.

fig.1) were used. These fractions did not form precipitates even with 0.3 mg antigen. The indirect immunofluorescence test with electrophoretically separated nucleosomal histones, showed that they contained antibodies against the nucleosomal calf-liver histones (not shown). As would be expected they reacted equally well with all 4 sheep nucleosomal histones (fig.2). The antibodies bind to core histones only and to no other protein of the sheep chromatin. No binding was observed with preimmune sera. It should be noted that the results with blotted histones were much better than those with the gels, where due to the long washing procedure the histone bands became diffuse and H2a was often lost. These data allow the conclusion that the rabbit IgG fraction contains specific antibodies against all 4 nucleosomal histones.

To further characterize the proteins of the ram sperm the anti-histone rabbit IgG were titrated by the microcomplement fixation test with variable amounts of calf liver nucleosomal histones (CLH) and with the sperm fractions S_1, S_2 and P_3: 0.3 μg of CLH gave a maximum of 88% complement fixation with 1:10 dilution of the rabbit anti-histone IgG (fig.3). The same dilution gives 85% MCF with 0.7 μg S_1 and 78% with 1 μg P_3. No complement fixation was obtained with the proteins of S_2.

These results indicate that some of the proteins in S_1 and P_3 contain histone antigeneic determinants. The increased amount of protein necessary for the

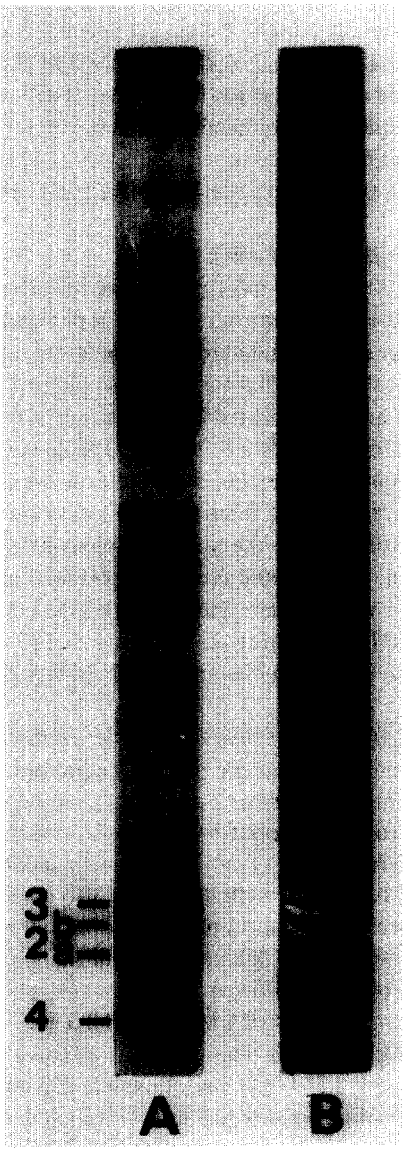

Fig.2. Indirect immunofluorescence staining of nucleosomal histones: (A) electrophoretic profile of total chromosomal proteins of sheep liver in 15% polyacrylamide gel [8], Coomassie brilliant blue staining; (B) another run of the same proteins as in (A) but transferred on nitrocellulose paper and reacted with anti-histone rabbit-IgG followed by FITC-labelled sheep anti-rabbit IgG.

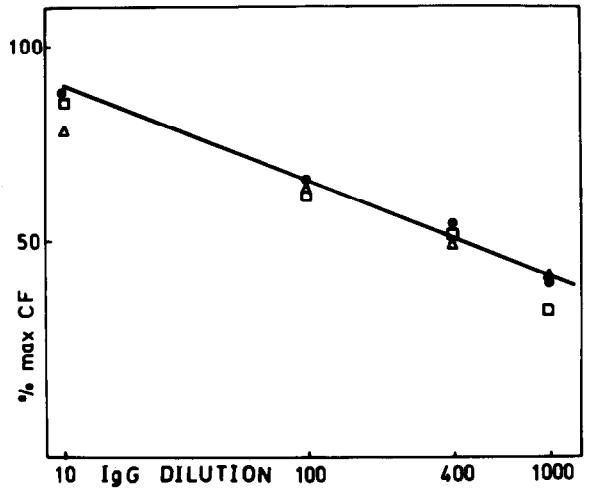

Fig.3. Microcomplement fixation test with sperm nuclear fractions S_1,S_2,P_3 and with calf liver histones (CLH). Dilution of the rabbit IgG (fractions 13,14, fig.1, containing ~0.7 mg protein/ml): 1/10 (●——●); 1/100 (○——○); 1/400 (▲——▲); 1/1000 (△——△).

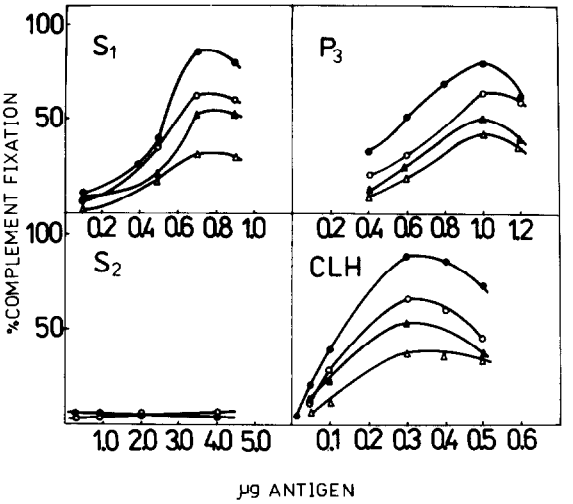

Fig.4. Maximal percentage of microcomplement fixation (MCF) as a function of the IgG dilution for sperm nuclear fractions S_1 (□——□), P_3 (△——△) and calf liver histones (CLH) (●——●).

heterologous reaction is due to the presence of non-histone proteins in S_1 and P_3 [2].

The presence of histone antigenic determinants in fraction P_3 was indicated also by a positive indirect immunofluorescence test (not shown) performed directly in the gel because of the very small amount of the histone-like proteins in this fraction. These data, however, could not show to what extent the proteins in P_3 were similar to histones or only shared some common antigenic determinants with them. To check this point we plotted the dependence of the maximal percentage of complement fixation on the logarithm of IgG dilution. All values obtained with both S_1 and P_3 lie close to the line of CLH (fig.4) which suggests a very high degree of similarity between these proteins [16]. Possible chemical or conformational modifications of the sperm histones may well account for the slight difference.

Fraction P_1, representing the DNA–protein complexes remaining after repeated extraction with salt and urea, gives also a positive reaction in the MCF test (not shown). This result shows that the presence of tightly bound histones is not an artifact of the SDS treatment.

These results show that although in small amounts, all 4 nucleosomal histones are present in the ram sperm nucleus, a fact which was indicated by the finding of 4 electrophoretic bands corresponding to the somatic nucleosomal histones [2]. The detection of histones in mammalian sperm nuclei is in agreement with data demonstrating histones in whole human spermatozoa by an immunofluorescence test [17] or in mouse sperm nuclei by a two-dimensional electrophoresis [3]. The existence of tightly bound histones resisting repeated extractions with 2 M NaCl, 6 M urea in the presence of 2-mercaptoethanol and even 2% SDS is a new and intriguing fact. A rough estimate of the amount of this tightly bound histone fraction gives ~2% of DNA (w/w). Bearing in mind the absence of somatic nuclei in 10^3 microscopically examined sperm nuclei this figure eliminates the possibility of significant histone contamination from somatic material.

Thus, our data not only prove the presence of somatic histones in mammalian spermatozoa but show in addition that histones in the sperm nucleus may occur in an unusual combination with DNA and/or other proteins which makes them resistant to the usual extraction procedures. Whether this is due to a covalent linkage to DNA or to the formation of a protein framework preventing its dissociation from DNA is unclear, but the fact that histone complexes are preserved and tightly fixed at some sites of the genome during spermiogenesis may have biological significance.

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