DNA-ASSOCIATED PROTEINS OF RAM SPERM NUCLEI

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1. Introduction

One of the basic, yet unsolved problems of molecular biology is whether the information necessary for cell differentiation resides in the primary structure of DNA alone (genetic mechanism), or is transmitted via nucleoprotein structures, possesing genetic continuity (epigenetic mechanism) [1–3].

The sperm cells of some mammalian and fish species present a situation which seems to contradict the possibility of epigenetic mechanisms. In these cells the somatic histones are replaced by highly basic, relatively small proteins of protamine type and it is believed that virtually only DNA is left as a carrier of information. Thus, it is widely accepted that the male DNA is a 'tabula rasa' upon which the pattern of histones and non-histone proteins may be re-established under the control of the cytoplasm of the fertilized egg only [4].

As an initial step in studying the mechanisms of informational transfer during spermatogenesis it is essential to know whether proteins of non-protamine type remain bound to DNA of mammalian sperm nuclei. Here we show that nuclei of mature ram spermatozoa, purified by treatment with detergents, contain proteins which remain associated with DNA after dispersion of the nuclei and removal of the protamines in salt–urea–thiol-containing solutions.

2. Methods

Fresh ram sperm was kindly supplied by the Institute of Biology and Immunology of Development and Reproduction (Sofia) and each batch was tested microscopically. Only vital and mature sperm samples were used. Fresh cells were washed 3 times with 10 mM Tris–HCl buffer (pH 8.0) containing 0.3 mM EDTA and 1 mM PMSF (phenylmethylsulphonylfluoride) as inhibitor of serine proteases. Disruption and elimination of acrosomes and tails from heads was achieved by sonication of the cells (twice for 3 min at 0°C) and centrifugation through 1 M sucrose as described for bull spermatozoa [5].

DNA was determined spectrophotometrically at 260 nm after acid hydrolysis (0.67 N $HClO_4$ for 20 min at 70°C) assuming that 1 A_{260}-unit corresponds to 38 μg hydrolyzed DNA. Protein was estimated by the Lowry method [6].

Protein fractions were analyzed by electrophoresis in acetic acid/urea polyacrylamide gels (PAG) [7] or SDS-containing PAG [8]. Molecular weights were roughly estimated by using a calibration curve of standard proteins with known relative molecular masses of 220–14 kM_r. Digestion with DNase I was performed in dialysis bags in the presence of 10 mM $MgCl_2$ as in [9].

Extraction of acid-soluble proteins was performed by treatment of the samples with 0.25 N H_2SO_4 for 30 min at 0°C, followed by centrifugation at 25 000 $\times g$ for 30 min. The acid-soluble proteins were collected from the supernatant by precipitation with 6 vol. acetone and vacuum-dried.

3. Results and discussion

In order to study the non-protamine proteins remaining bound to DNA after sperm maturation it was essential to use a method for the isolation of pure nuclei free of contaminating nuclei of somatic or immature cells. We took advantage of the extreme stability of the mature sperm nuclei to sonication [10,11] and to detergents which remove nuclear membranes and immature sperm cells [11,12]. To disrupt and remove possible contaminating nuclei, the soni-

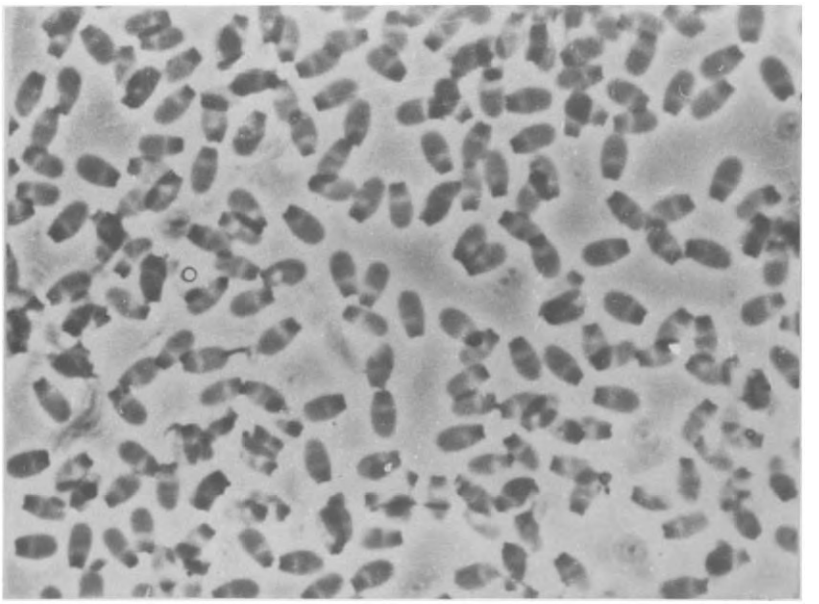

Fig.1. Sonication-resistant sperm nuclei after treatment with 1% SDS (×450).

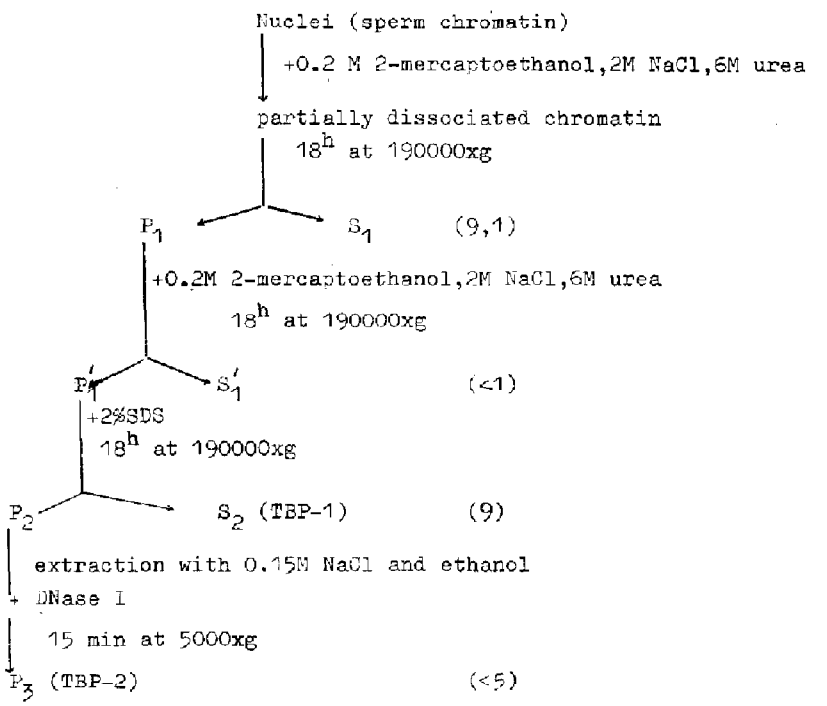

Fig.2. Flow-diagram of the preparation procedure of proteins associated with ram sperm DNA. The figures in brackets show the quantity of the protein fractions, expressed as % of the DNA in weight units.

cation-resistant sperm heads were treated with 1% SDS aqueous solution for 90 min at room temperature. The compact, membrane-free nuclei were collected by centrifugation through 1 M sucrose for 10 min at 600 × *g* (fig.1). Virtually the same results were obtained when sperm nuclei were prepared by treatment of the heads with 1% Triton X-100 and 0.1 mM Na-deoxycholate as described for bull sperm nuclei [13].

It was found that in the absence of proteolysis (due to the presence of PMSF) dissociation of protamines and partial unfolding of DNA could be achieved only by the combined action of NaCl, urea and 2-mercaptoethanol (fig.2). This is in agreement with the results obtained for bull sperm nuclei [13]. The partially disssociated chromatin was subjected to fractionation according to the scheme in fig.2.

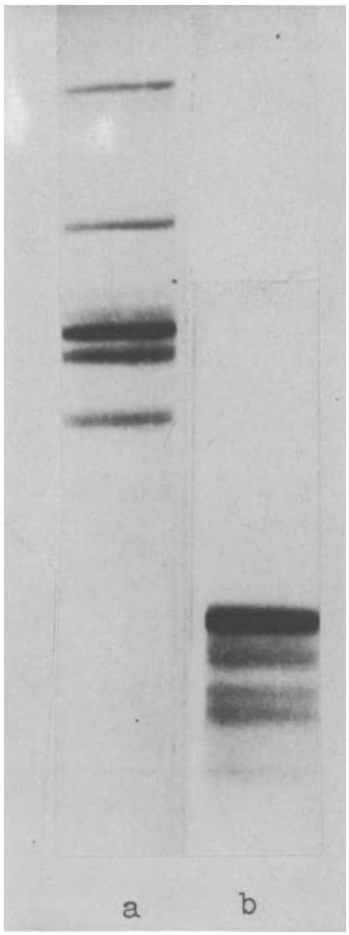

Fig.3. Acetic acid/urea PAG electrophoregrams of: (a) ram sperm protamines; (b) rat liver somatic histones.

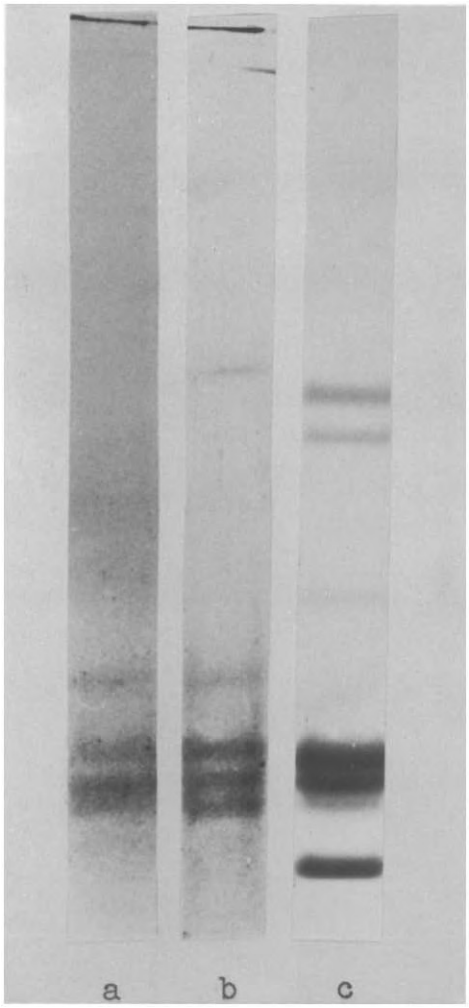

Fig.4. Electrophoregram of: (a) S_1 proteins (see fig.2) in SDS–PAG; (b) the acid extractable proteins of S_1; (c) rat liver somatic histones.

Fraction S_1 (fig.2) contained the sperm protamines. They were analyzed by electrophoresis in acetic acid/urea PAG [7], which showed 5 fractions moving faster than the somatic histones (fig.3). In view of the presence of PMSF it is not likely that the heterogenity observed is due to proteolysis. Such a heterogeneity of the basic sperm protamines was found in other species [14–16]. The ram sperm protamines could not be analyzed by SDS–PAG electrophoresis since they precipitated in the SDS-containing buffers as noted [14,17,18]. The sample buffer used by Laemmli [8], however, solubilized some of the proteins of fraction S_1 which were fractionated in SDS–PAG (fig.4). Several weak bands

with electrophoretic mobilities similar to that of the somatic nucleosomal histones were obtained. These proteins were acid-soluble and their fractionation after extraction with 0.25 N H_2SO_4 is shown in fig.4b. Although our data do not prove that these proteins are somatic histones, their electrophoretic mobilities and acid solubility suggest that some somatic histones may have been preserved in the mature sperm nuclei. A better resolution and characterization of these histone-like proteins was not possible due to their small quantity in the presence of large amounts of protamines which overloaded the gels. The same holds true for acetic acid/urea gels where faint bands in the region of histones could be revealed only after heavy overloading of the gels.

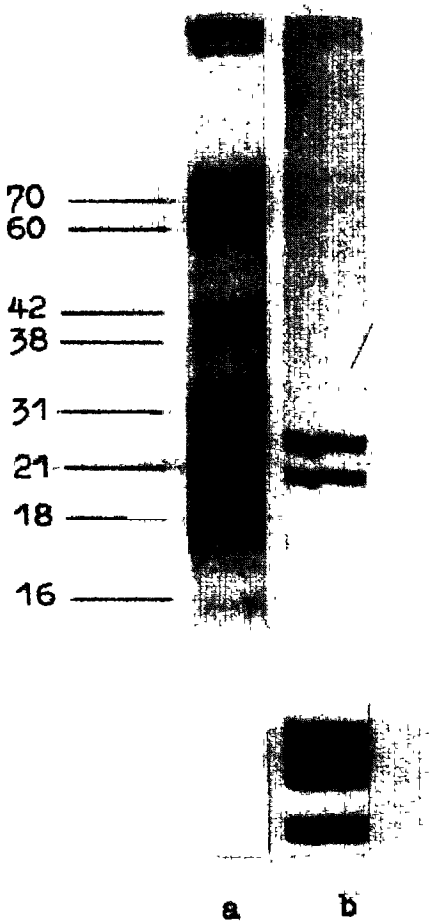

Fig.5. Electrophoregrams of: (a) S_2 proteins (TBP-1 of fig.2) in SDS–PAG (figures indicate M_r); (b) rat liver somatic histones.

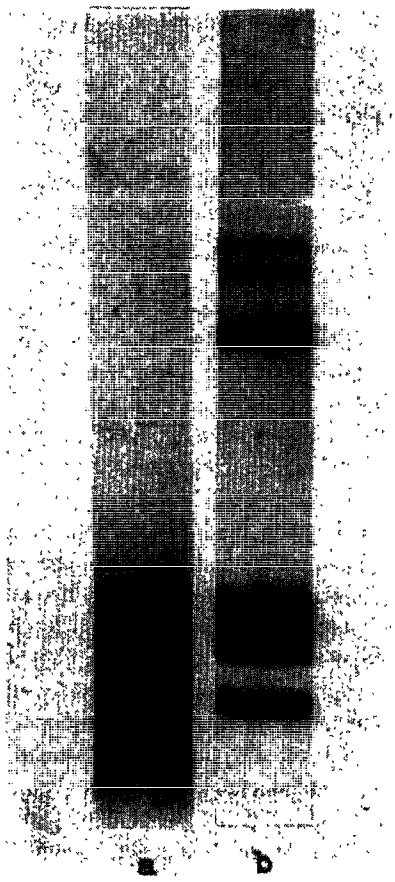

Fig.6. SDS-PAG electrophoregrams of: (a) proteins obtained after DNase I digestion of DNA in the P_2 fraction (see fig.2); (b) rat liver somatic histones.

The pellet after the first centrifugation (P_1 in fig.2) was re-extracted with 2-mercaptoethanol, salt and urea for the complete removal of the protamine-type proteins. This resulted in the release of $\leqslant$10% of the protamines extracted in S_1. The pellet P_1' thus obtained, was dispersed in 2% SDS. As shown in fig.5 some non-histone proteins still remain associated with the sperm DNA after the salt–urea–thiol extraction and can be released from it only after treatment with SDS. These tightly bound proteins (TBP-1) are 16–70 kM_r and are acid insoluble.

It is interesting to note that the pellet P_2 (see fig.2) still contains some proteins which are not released from DNA after all procedures described thus far. Heating of P_2 in the sample buffer [8] and electrophoresis in SDS–PAG revealed that the protein material remained at the gel starts and no protein fractions could be detected in the gels (not shown). These pro-

teins (TBP-2) were released from DNA only after digestion of the latter by DNase I, which shows that they are firmly bound to DNA. They were collected by low-speed centrifugation and analyzed on SDS–PAG (fig.6). It should be noted that a significant amount of the material still remained at the start, but nevertheless, some fractions were present which gave a similar electrophoretic pattern to that of somatic nucleosomal histones.

These observations show that purified ram sperm nuclei contain a considerable amount of proteins of non-protamine type, which remain with DNA after dispersal of the nuclei and removal of all protamines. These proteins are most probably bound to DNA and can be subdivided into proteins that dissociate from DNA in 2% SDS (TBP-1) and proteins that remain bound to DNA in 2% SDS (TBP-2). Since TBP-2 can be dissolved in SDS and electrophoresed only after an extensive digestion of DNA it may be thought that these proteins are covalently bound to DNA but the proof requires further work. Both TBP-1 and TBP-2 are heterogeneous in relative molecular mass.

It is noteworthy that TBP-2 contain a group of proteins with electrophoretic mobilities of the somatic nucleosomal histones. In fact it has never been shown that the replacement of the somatic histones by protamines is complete. Non-protamine proteins found thus far in the sperm nuclei of mammals were believed to be due to contaminations [15,19]. Recently, however, somatic histones were detected in mature human sperm nuclei by immunofluorescence [20,21].

Tightly bound proteins have been found in many tissues [9,21–24]. However, different authors use different criteria for evaluating the strength of the protein binding to DNA and the results are not readily comparable. In some cases it has been suggested that part of these proteins are attached to DNA by covalent bonds but the exact nature of these bonds remains obscure.

The biological significance of the tightly bound proteins is not clear. It may be speculated that these proteins mark some regions in DNA ensuring the transfer of structural information. One possibility is that they maintain a special level of organization of chromatin into genetic domains (large groups of genes that can be switched on and off as a whole), which may correspond to the structural domains found in DNA [25,26].

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