Changes in nuclear content of protein conjugate histone H2A–ubiquitin during rooster spermatogenesis

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Electrophoretic analysis of acid-soluble chromosomal proteins isolated from rooster testis cell nuclei at different stages of spermatogenesis, revealed that the nuclear content of a protein identified by its solubility, electrophoretic mobility and amino acid analysis as the protein conjugate histone H2A–ubiquitin (uH2A, A24) changed markedly from meiotic cells to late spermatids. The protein was not detectable in tetraploid primary spermatocytes; it was present in 1.7% of the total amount of nucleosomal core histones in early spermatids and reached its maximum level (3.5% and 11%) at the end of spermiogenesis, when histones are replaced by the protamine galline.

Histone H2A *Ubiquitin* *uH2A* *A24* *Chromatin* *Spermatogenesis*

1. INTRODUCTION

The covalent conjugate histone H2A–ubiquitin (uH2A or A24) is a branched protein resulting from the post-translational addition of the non-histone protein ubiquitin to histone H2A at lysine 119 [1].

One or both of the H2A molecules in the same nucleosome can be converted to uH2A. This modification has little influence on chromatin structure at the level of individual nucleosomes [2]. However, ubiquitin binding to the core histone H2A may prevent formation of higher order chromosomal structures by modifying nucleosome–nucleosome interactions [3,4].

Spermatogenesis offers an excellent model for investigating a possible role of the covalent conjugate uH2A in the structural and functional changes that chromatin undergoes throughout the differentiation process [5–7];

(i) Tetraploid primary spermatocytes possess transcriptionally active meiotic chromosomes;

(ii) Premeiotic and postmeiotic cells as spermatogonia and early spermatids contain transcriptionally active interphasic chromatin;

(iii) The chromatin of spermatids undergoing differentiation to spermatozoa (spermiogenesis) becomes relaxed, exposing binding sites on DNA, prior to its final condensation as a consequence of the replacement of histones by a highly basic protein, the protamine galline.

2. EXPERIMENTAL

2.1. *Isolation and separation of rooster testis cell nuclei*

Hubbard White Mountain roosters (25–50 weeks old) were used throughout this study. Nuclei were isolated from fresh rooster testes and separated by sedimentation at unit gravity by the procedure in [5], except for the buffer used: 10 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$ and 25 mM KCl, 0.14 mM spermidine. Just before use, $NaHSO_3$ was added to 50 mM final conc. without further adjustment of the pH.

EDTA-resistant spermatid nuclei were prepared from purified nuclei by 0.02 M EDTA treatment

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followed by suspension in solutions of decreasing ionic strength (10 mM and 2 mM Tris–HCl, pH 7.5) and homogenization at full speed for 5 min in a Sorvall omnimixer equipped with a micro attachment.

2.2. *Analysis of acid-soluble nuclear proteins*

Acid-soluble nuclear proteins (0.2 M H_2SO_4) were analyzed electrophoretically on 15% polyacrylamide slab gels as in [8] on exponential polyacrylamide–SDS gels (10–16%) as in [9], or in two-dimensional gel electrophoresis (acetic acid–urea followed by SDS). The gels were scanned with a 2410 Gilford linear-transport scanner. The protein conjugate uH2A was obtained by preparative SDS–polyacrylamide gel electrophoresis (3-mm gels). The gel was stained with Coomassie blue and the putative uH2A band excised, extracted and the dye removed as in [10]. Amino acid analyses were done using a Beckman 119C amino acid analyzer, after hydrolysis of the samples in 6 N HCl at 110°C for 24 h. No corrections were made for hydrolytic losses.

3. RESULTS

3.1. *Electrophoretic separation of acid-soluble nuclear proteins obtained from rooster testis cell nuclei*

The electrophoretic separation patterns of acid-soluble nuclear proteins of rooster testis cell nuclei are shown in fig.1. In addition to histones an extra band of lower mobility than histone H1 in acetic acid–urea–polyacrylamide gels appeared (fig.1a). This band, previously designated X [5], was not coextracted with histone H1 in 5% perchloric acid (fig.1b). Band X, purified by preparative acetic acid–urea–polyacrylamide gel electrophoresis, was resolved by SDS–electrophoresis in two major components of M_r 30000 and 27000 (fig.1e,f). Two-dimensional gel electrophoresis of acid-soluble nuclear proteins extracted from late spermatid nuclei also showed that band X (first dimension) was resolved in two components in the second dimension (fig.1c). The major component of band X was identified by its solubility, electrophoretic mobility and amino acid analysis as the protein conjugate ubiquitin–H2A (fig.1, table 1).

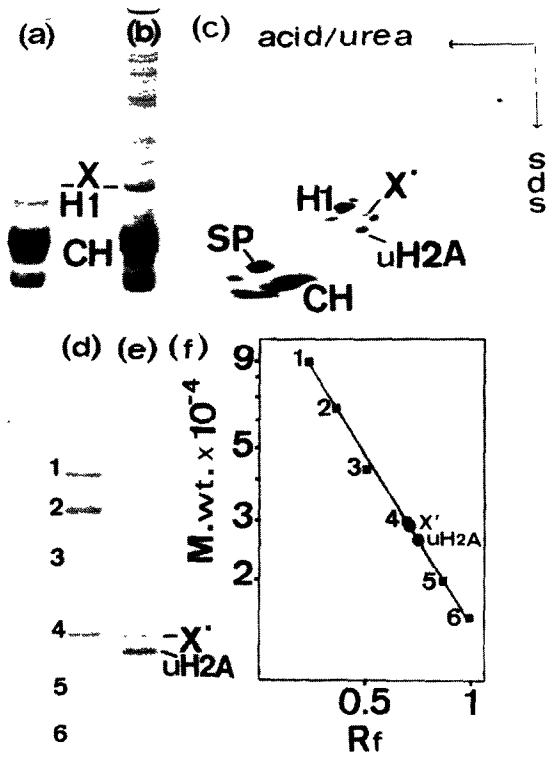

Fig.1. (a) Acid–urea gel electrophoresis of 0.2 M H_2SO_4-soluble proteins from rooster testis cell nuclei; (b) acid–urea gel electrophoresis of 0.2 M H_2SO_4-soluble proteins from rooster testis cell nuclei after treatment with 5% perchloric acid; (c) two-dimensional gel electrophoresis of 0.2 M H_2SO_4-soluble proteins from late spermatid nuclei; (d) standard markers – (1) phosphorylase *b*, (2) bovine serum albumin, (3) ovalbumin, (4) carbonic anhydrase, (5) soybean trypsin inhibitor, (6) α-lactalbumin; (e) SDS–gel electrophoresis of band X purified by preparative electrophoresis on acid/urea gels; (f) M_r determination of X′ and uH2A on SDS–gel electrophoresis; CH, core histones; SP, spermatidal protein.

3.2. *Changes in nuclear content of protein uH2A throughout rooster spermatogenesis*

We have shown that band X increased in density during rooster spermiogenesis [5]. Its major component, uH2A, was not detectable in tetraploid primary spermatocyte nuclei (stage I), it was barely detectable in a fraction containing nuclei of small primary spermatocytes, secondary spermatocytes and spermatogonia cells (stage II), it represented 1.7% of the total nucleosomal core histones in

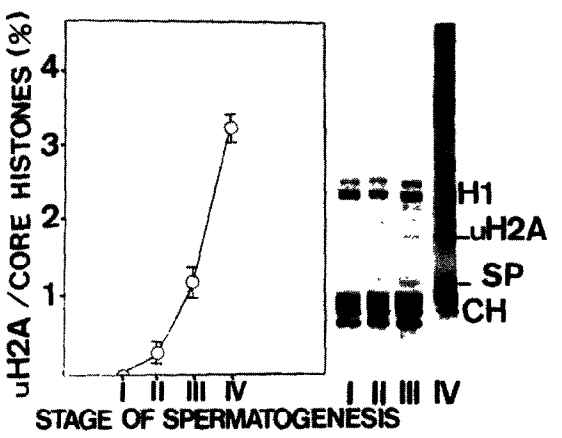

Fig.2. Changes in nuclear content of protein uH2A during rooster spermatogenesis and SDS–gel electrophoretic analysis of 0.2 M H_2SO_4-soluble nuclear proteins from different stages of rooster spermatogenesis: stage I, tetraploid primary spermatocytes; stage II, small primary spermatocytes, secondary spermatocytes and spermatogonia; stage III, early spermatids; stage IV, late spermatids and testicular spermatozoa; CH, core histones; SP, spermatidal protein.

Table 1

The amino acid composition (% mol) of rooster testis uH2A compared with the amino acid analyses of calf thymus uH2A [11] and the expected H2A plus ubiquitin conjugate

Amino acids	Rooster testis	Calf thymus	H2A + ubiquitin
Trp	0.0	0.0	0.0
Lys	9.4	11.3	10.3
His	2.1	2.4	2.4
Arg	6.4	7.4	7.9
Asx	8.3	7.3	7.3
Thr	5.0	6.5	5.9
Ser	8.0	4.5	3.4
Glx	14.6	12.3	11.8
Pro	5.4	5.6	3.9
Gly	9.4	9.2	8.9
Ala	8.4	9.6	9.4
Val	5.9	4.9	5.9
Met	2.5	0.3	0.5
Ile	3.6	5.8	6.4
Leu	6.5	10.9	12.3
Tyr	1.8	1.3	2.0
Phe	2.4	0.9	1.5

nuclei of early spermatids (stage III) and reached a 3.5% in late spermatid nuclei (stage IV) as shown in fig.2.

In EDTA-resistant spermatid nuclei, at the end of spermiogenesis when nucleosomal core histones are almost completely replaced by protamine, the percentage of the protein uH2A in relation to the remaining core histones was in the 10–12% range. Protein X was not present in rooster spermatozoa where nucleohistone has been completely replaced by nucleoprotamine [5].

Similar results were obtained when cells, instead of nuclei, were fractionated by sedimentation at unit gravity (not shown).

4. DISCUSSION

The nuclear protein uH2A was not detectable in rooster tetraploid primary spermatocytes. The removal of ubiquitin from nucleosomal core uH2A may be essential for the assembly of chromatin fibers into meiotic chromosomes during the long prophase of tetraploid primary spermatocytes. Removal of ubiquitin from uH2A occurs during the interphase–mitosis transition [3].

The ratio uH2A/nucleosomal core histones increased during rooster spermiogenesis. An exposition of binding sites on DNA in late spermatid nuclei has been demonstrated by their high capacity for binding actinomycin D and their increased number of initiation sites for RNA synthesis in vitro [5,6]. The exposition of DNA binding sites during spermiogenesis correlates with hyperacetylation of histone H4 and high turnover of its acetyl groups [7]. The high content of uH2A during rooster spermiogenesis favors the hypothesis that ubiquitin binding to histone H2A is a mechanism for chromatin relaxation [3,4]. Relaxation of chromatin fibers as a consequence of the attachment of ubiquitin to histone H2A and histone H4 hyperacetylation at the end of spermiogenesis would enable the binding of protamine to the accessible sites on DNA.

The functional role of protein uH2A is controversial. Both activation and inhibition of transcriptional activity have been postulated [3,4,12–14]. The lack of uH2A in transcriptionally active meiotic chromosomes as well as its high content in late spermatids with transcriptionally inactive relaxed chromatin suggests that uH2A plays a

structural role not univocally related with transcriptional activity.

We do not yet know whether uH2A may be instrumental in the loss of nucleosomal organization at the end of spermiogenesis when histones are replaced by protamine. Protein–ubiquitin conjugates in the cytoplasm are substrates for an ATP-dependent proteolytic system [15]. Further studies on the turnover of uH2A in late spermatids will ascertain if a similar mechanism could operate for histone removal at the end of spermiogenesis.

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