Marked Differences in the Ability of Distinct Protamines To Disassemble Nucleosomal Core Particles in Vitro†

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ABSTRACT: In accordance with the results of classical experiments performed in vitro with calf thymus chromatin and the fish protamine salmine, we have observed that this highly basic, small molecular weight protamine cannot cause major displacement of histones from nucleosomal core particles at concentrations several times higher than physiological (arginine/nucleotide ratios 1–8) and that hyperacetylation of histones facilitates nucleosome disassembly. However, the avian protamine galline, with molecular weight and number of arginine residues almost twice those of common fish protamines, is able to displace the nucleosomal core histones from DNA in vitro at concentrations (arginine/nucleotide ratios 0.6–1.2) within the physiological range (0.8). Our results suggest that the binding of the avian protamine galline to chromatin could be directly involved in the rapid disassembly of nucleosomes that takes place during the nucleohistone nucleoprotamine transition in chicken spermiogenesis.

The most marked changes in chromatin structure observed in eucaryotes take place when nucleosomes are disassembled and replaced by a highly condensed nucleoprotein complex. This transition occurs during spermiogenesis, when histones are removed from DNA and replaced by protamines (Mezquita, 1985) and also when a similar process takes place during the transition from viral DNA, organized in nucleosomal complexes, to the condensed nucleoprotein cores released from virions (Alestrom et al., 1984).

Protamines are highly basic proteins isolated from the nuclei of spermatids and spermatozoa of many species (Ando et al., 1973). The molecular weights of common fish protamines range below 5000. The polypeptide chain is longer in avian protamines (65 amino acids in galline, molecular weight 9829) (Nakano et al., 1976). The fish protamines salmine Al, iridine Ib, and clupeine Z contain 20, 22, and 21 arginine residues, respectively, and do not contain tyrosine (Figure 1). The rooster protamine galline contains 38 arginine residues and four residues of tyrosine (Nakano et al., 1976). The number of arginine residues present in the C-terminal region of the protamine galline greatly exceeds the number found in fish and mammalian protamines (Figure 1). Viral core proteins are remarkably similar to protamines, and their functions may resemble those of protamines (Dixon et al., 1985).

The mechanisms of histone removal and nucleosome disassembly during the nucleohistone–nucleoprotamine transition at the end of spermiogenesis and during the transition from viral nucleosomal particles to the condensed viral nucleoprotein core complexes are unknown at present. The inefficiency of the fish protamine salmine to remove histones from calf thymus chromatin in vitro together with the observation that transforming trout sperm chromatin contains small basic peptides, which may be proteolytic products of histones, led to the proposal that the displacement of histones from chromatin in spermatids could be achieved by the action of a protease firmly associated with chromatin (Marushige & Dixon, 1969). The protease would hydrolyze preferentially acetylated histones (Wong & Marushige, 1975; Marushige & Marushige, 1983).

In this paper we show that the avian protamine galline is able to displace completely the nucleosomal core histones from DNA in a range of arginine/nucleotide of 0.6–1.2 (physiological ratio 0.8). Our results suggest that the binding of the protamine galline to chromatin in vivo can be directly involved in the disassembly of nucleosomes at the end of the spermiogenesis.

Experimental Procedures

Separation of Testicular Cells at Unit Gravity. Cell suspensions were prepared and separated by sedimentation at unit gravity by the procedure previously described (Oliva et al., 1982). Mature testes from Hubbard White Mountain roosters (25–30 weeks old) were decapsulated and minced finely with scissors. The minced tissue was gently suspended in 10 volumes of minimum essential medium (Eagle) containing 0.1% (w/v) trypsin and 2 μg of DNase I/mL. The suspension was incubated at 31 °C for 30 min with gentle stirring in a water bath. After incubation, the cell suspension was filtered through four layers of surgical gauze and centrifuged for 20 min at 1500*g* in a JS-7.5 Beckman rotor. The sample was resuspended in 50 mL of Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 0.02% (w/v) soybean trypsin inhibitor, 0.1% bovine serum albumin, and 0.1% glucose. Sedimentation chambers with a diameter of 28 cm were used. The cell suspension was diluted in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline to a final concentration of 16×10^6/mL, and 80 mL of the sample was loaded through a buffered step gradient of glycerol (1.5%/3%/6% w/v).

Spermatozoa obtained from the vas deferens sedimented with sedimentation velocities between 0.5 and 1 mm/h. Elongated spermatids showed sedimentation velocities between 1 and 3 mm/h. Round spermatids sedimented in the region 2–4 mm/h. Meiotic and premeiotic cells sedimented with higher sedimentation velocities (>4 mm/h) according to their differences in size (Oliva et al., 1982).

Labeling of Testicular Cells with [^{3}H]Acetate. Testicular cells were labeled with [^{3}H]acetate as previously described (Mezquita et al., 1982). The cell suspension was incubated with 2 mCi of [^{3}H]acetate (Amersham; specific radiactivity 300 mCi/mmol) at 31 °C for 30 min in the presence of cy-

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				Total Arg
Galline				
ARYRSRGRSRSRRT	RRRRSPRSGRRRSPRRRRSRRR	RRYGSARRSRRSGGVRRRRYGSRRRRRRY		
7	15	16		38
Salmine A1				
PRRRRSSSRPVRRR	RRPRVSRRRRRRGGRRRR			
7	13			20
Iridine Ib				
PRRRRRRSSSRPIR	RRRPRRVSRRRRRGGRRRR			
8	14			22
Clupeine Z				
ARRRRSRRASRPVR	RRRPRRVSRRRRARRRR			
8	13			21
Mouse 1				
ARYRCCRSKSRSRC	RRRRRRCRRRRRRCCRRRRRRC	CRRRRSYTIRCKKY		
5	18	5		28
Ram				
ARYRCCLTHSRSRC	RRRRRRRCRRRRRRFGRRRRRR	VCCRRYTVVRCTRQ		
4	19	4		27
Human P1				
ARYRCCRSQSRSRY	YRQRQRSRRRRRRSCQTRRRAM	RCCRPRYRPRCRRH		
5	12	7		24

FIGURE 1: Sequence comparison of the protamines galline (Nakano et al., 1976), salmine Al, iridine Ib, clupeine Z (Ando & Watanabe, 1969; Ando et al., 1973), mouse protamine 1 (Kleene et al., 1985), ram protamine (Sautiere et al., 1984), and human protamine P1 (McKay et al., 1985) in one-letter notation: A, alanine; C, cysteine; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.

cloheximide added to a final concentration of 0.2 mM.

Isolation and Analysis of Histones and Protamine. Nuclei from rooster testis cells were isolated by the citric acid procedure as described in Krause (1978), and nuclear basic proteins, histones, and protamines were extracted in 0.3 M HCl, precipitated with 20% trichloroacetic acid, and washed with acidified acetone and acetone (Johns, 1977). The protamine galline free of histones was obtained from nuclei isolated with citric acid (Mezquita & Teng, 1977a), containing 10 mM benzamidine, from vas deferens spermatozoa. Electrophoresis was performed either in SDS[1]–polyacrylamide gels (Thomas & Kornberg, 1978) or in acetic acid/urea/Triton/polyacrylamide gels containing 7.5 M urea and 6 mM Triton X-100 as described by Zweidler (1978). Gels were stained with Coomasie blue/formaldehyde (Irie & Sezaki, 1983) and scanned with a Model 2410 Gilford linear transport scanner at 600 nm. The methods of Bonner and Laskey (1974) and Laskey and Mills (1975) were used to prepare gels for fluorography of the ^{3}H-acetylated histones. Presensitized Fuji X-ray film was exposed at −70 °C for 15 days.

Preparation of Nucleosomal Core Particles. Nucleosomal core particles were prepared as described by Bode et al. (1983). Rooster testis cell nuclei (38 mg of DNA) were washed twice with 50 mL of a buffer containing 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM butyrate, 0.2 mM PMSF, and 5 mM Tris-HCl (pH 8.0). After centrifugation at 2000*g* for 10 min, nuclei were resuspended in 18 mL of a buffer containing 0.25 M sucrose, 0.1 mM $CaCl_2$, 1 mM butyrate, 0.2 mM PMSF, and 5 mM Tris-HCl (pH 8.0). After equilibration for 15 min at 0 °C, nuclei were preincubated for 2 min at 37 °C and then the buffer was supplied with 1800 units of micrococcal nuclease. After 2 min, the mixture was cooled in ice, adjusted to 0.15 M with NaCl, and centrifuged at 2000*g*, 5 min at 0 °C. The supernatant (S1) contained nucleosomal core particles enriched in nonhistone proteins. The pellet resuspended in 18 mL of the same buffer was subjected to a second digestion for 5 min and similarly fractionated to yield a second supernatant (S2) containing a monomer fraction depleted of nonhistone proteins and enriched in acetylated histones. The supernatants S1 and S2 were adjusted to 1 mM EDTA and the nucleosomal core particles further purified by preparative electrophoresis under nondenaturing conditions.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

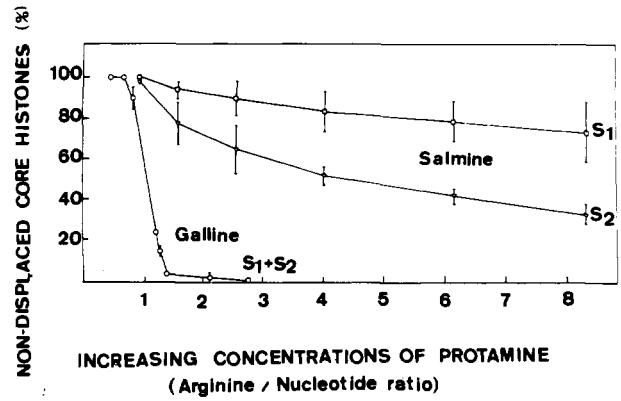

FIGURE 2: Nucleosome disassembly in vitro by the protamines salmine and galline: displacement of histones from nucleosomal core particles (fractions S1 + S2) by the protamine galline (○); displacement of histones from nucleosomal core particles, fraction S1 (□) and fraction S2 (▽), by the protamine salmine. Nucleosome core particles, fractions S1 and S2, were prepared as described under Experimental Procedures.

Electrophoresis of Nucleosomal Core Particles on Nondenaturing 4% Polyacrylamide Gels. Electrophoresis was performed on nondenaturing 4% polyacrylamide gels as described by Bode (1984) with the following modifications: A preparative gel (14 cm wide, 5 cm high, 2 cm thick) was used, and 15 mL of S1 or S2 samples was loaded. An elution chamber 0.6 mm high was placed at the bottom of the gel, and a buffer containing 44.5 mM Tris–borate, 44.5 mM boric acid, and 4 mM EDTA (pH 8.3), flowing through the elution chamber during electrophoresis, was continuously monitored, measuring the absorbance at 260 nm. The monomeric core particles were identified by DNA length and protein composition.

Protamine-Mediated Histone Displacements. Nucleosomal core particles (20–70 μg of DNA/mL) were treated with increasing amounts of protamine in 0.2 M NaCl, 1 mM NaEDTA, and 5 mM Tris-HCl, pH 8.0. After 4 h of incubation at 0 °C, with Vortex mixing at intervals, samples were centrifuged at 24000*g* for 10 min and both, pellets and supernatans, were used for histone and DNA quantification.

Analytical Methods. DNA was determined by the diphenylamine reaction (Burton, 1968) using calf thymus DNA as standard. Protamines were quantified by the Sakaguchi reaction (Dubnoff, 1941).

RESULTS

Displacement of Nucleosomal Core Histones by the Protamines Galline and Salmine in Vitro. When nucleosomal core particles obtained as described under Experimental Procedures (fractions S1 + S2) were treated with increasing amounts of the protamine galline, all four nucleosomal core histones were progressively liberated by the protamine within a range of protamine concentrations between 0.6 and 1.2 for the arginine/nucleotide ratio (Figure 2). The protamine galline is able to disassemble nucleosomal core particles obtained from rooster testis and chicken erythrocyte nuclei in the same range of protamine concentrations. The displacement of histones from nucleosomal core particles of chicken erythrocyte nuclei is independent of the methodology used to isolate nuclei. The same results were obtained with citric acid or at neutral pH for the purification of chicken erythrocyte nuclei (results not shown). The lysine-rich histones H2A and H2B are prefer-

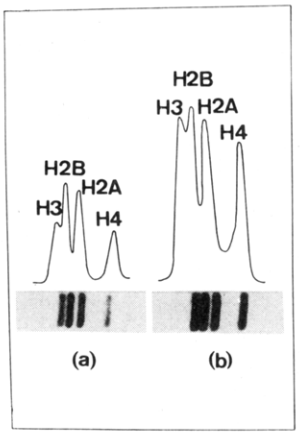

FIGURE 3: Histone-extracted incubating nucleosome core particles obtained from chicken erythrocyte nuclei (citric acid procedure) with two different concentrations of the protamine galline: arginine/nucleotide ratios 1.0 (a) and 1.6 (b). Electrophoreses were performed in SDS–polyacrylamide gels as described under experimental Procedures.

entially displaced in relation to the arginine-rich histones H3 and H4 at low concentrations of protamine (Figure 3). The protamine salmine is much less efficient than galline in the removal of histones (Figure 2). No major displacement of histones from nucleosomal core particles (fraction S1) was accomplished by salmine in a range of arginine/nucleotide ratios of 1–8. Acetylation of histones (fraction S2) facilitates their displacement by the protamine salmine (Figure 2).

Correlation between Histone Hyperacetylation, Histone Removal, and Replacement of Histones by the Protamine Galline during Rooster Spermiogenesis. Basic proteins isolated from rooster testis spermatid nuclei at successive stages of spermiogenesis were quantified after electrophoresis in acetic acid urea gels. Decreasing levels of nucleosomal core histones and increasing amounts of the protamine galline were detected as spermiogenesis proceeded (Figure 4). The level of hyperacetylated species of histone H4 in relation to the nonacetylated or monoacetylated forms rose markedly during spermiogenesis simultaneously with the protamine binding (Figure 4). Tetraacetylated and triacetylated H4 increased during spermiogenesis, while the monoacetylated and the diacetylated H4 forms decreased (Figures 4 and 5). The degree of hyperacetylation observed during spermiogenesis, particularly of histone H4, is comparable to that obtained after inhibition of histone deacetylase by butyrate in other systems (Figure 5).

Discussion

Our results, in accordance with previous observations (Wong & Marushige, 1975; Bode et al., 1977, 1980) show that the small molecular weight fish protamines cannot cause major displacement of histones in vitro in a range of arginine/nucleotide ratios of 1–8 and that hyperacetylation of histones increases the efficiency of histone removal by the fish protamines. Although the arginine/nucleotide ratio necessary for the protamine-mediated displacement of histones in vitro changes using different temperature and salt conditions (Evans et al., 1970), total removal of histones from chromatin by fish protamines only can be accomplished with arginine/nucleotide ratios several times higher than physiological. However, the avian protamine galline, with a molecular weight and number of arginine residues (38 per molecule) almost twice that of common fish protamines (21 in salmine), is able to displace completely nucleosomal core histones from DNA in a range of arginine/nucleotide of 0.6–1.2 (ratio arginine/nucleotide

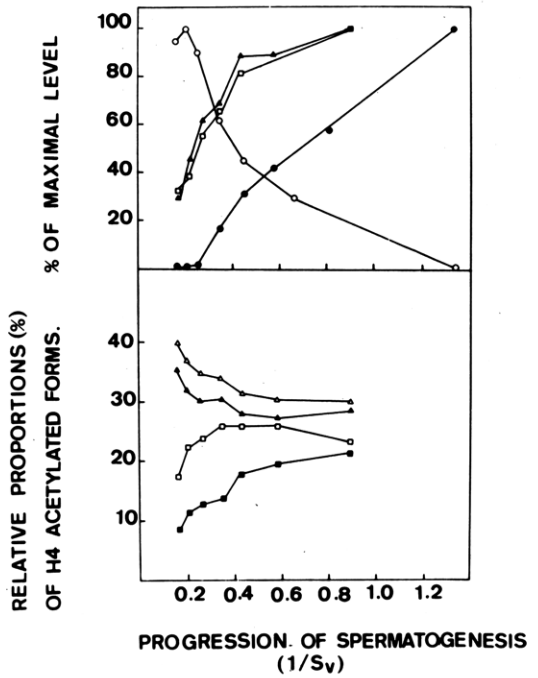

FIGURE 4: Changes in nuclear basic proteins at successive stages of rooster spermiogenesis. (Top) Quantitative changes in nuclear basic proteins, histones (○) and protamine (●). The ratio of histones/DNA of 1 (w/w), determined in meiotic and premeiotic cells, is referred as 100% nucleohistone. The ratio of arginine/nucleotide of 0.8, determined in spermatozoa from the vas deferens, is referred as 100% of nucleoprotamine. Changes in the relative proportions of the acetylated forms of histone H4: ratio of triacetylated/monoacetylated H4 (▲); ratio triacetylated/nonacetylated H4 (□). (Bottom) Relative proportions of [^{3}H]acetate incorporated into histone H4. The proportions were determined from the fluorograms of acetic acid/Triton X-100/urea gels as described under Experimental Procedures. Monoacetylated (▲), diacetylated (△), triacetylated (□), and tetraacetylated (■) histone H4. Meiotic and premeiotic cells, 1/Sv = 0.17–0.19; round spermatids, 1/Sv = 0.21; elongated spermatids at successive stages of spermiogenesis, 1/Sv = 0.22–0.9.

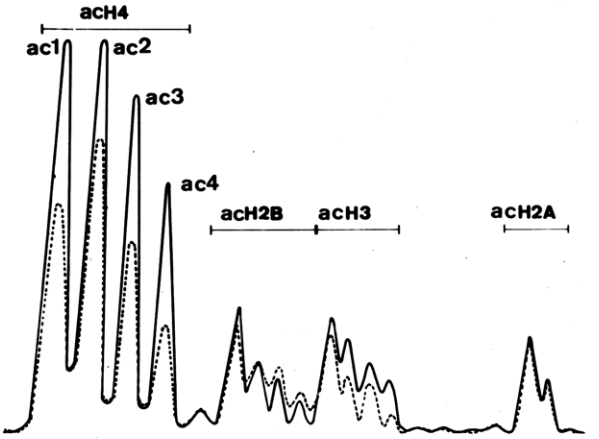

FIGURE 5: Acetylation of histone during rooster spermiogenesis. Densitometric scans of fluorograms of acetylated histones extracted from elongated spermatids (—) and round spermatids (---). Histones were extracted from purified nuclei and electrophoresed in acetic acid/urea/Triton X-100/polyacrylamide gels as described under Experimental Procedures.

in rooster spermatozoa 0.8). No major differences were observed in the efficiency of histone removal by the protamine galline when nucleosomes with different degrees of acetylation were used for the experiments of nucleosome disassembly.

We interpret these results in the sense that both the number of arginine residues per molecule of protamine bound to a segment of DNA and the degree of acetylation of histones can

modulate the disassembly of nucleosomes. The arginine residues of the protamine can establish multiple hydrogen bonds and electrostatic interactions with the phosphate oxygens of DNA with significantly higher affinity than the interactions established by lysine and histidine residues of the nucleosomal core histones (DeSantis et al., 1974), especially when the positive charges of the lysine residues of histones are neutralized by acetylation.

The marked differences observed between fish and avian protamines in the number of arginine residues per molecule and in the relative efficiency of nucleosome disassembly in vitro correlate with the time required for the nucleohistone–nucleoprotamine transition in both species. The spermiogenesis is a rather slow process in fishes (several weeks) and appreciably faster in birds (6 days in the chicken).

Histone hyperacetylation may contribute to the opening of chromatin structure for protamine binding (Mezquita & Teng, 1977b; Oliva & Mezquita, 1982). This chemical modification has not been observed in species that retain histones in the sperm nucleus (Ruiz-Carrillo & Palau, 1973; Kennedy & Davies, 1980) and is present in the spermatidal chromatin of trout, rat, and chicken during the replacement of nucleosomal histones by protamine (Christensen & Dixon, 1982; Grimes & Henderson, 1984; Oliva & Mezquita, 1982). It has been suggested that the level of acetylation of lysine residues per nucleosome during spermiogenesis (8–10 residues) would be sufficient to open up the nucleosome (Bode et al., 1983; Christensen et al., 1984). However, it is not clear, at present, to what extent the hyperacetylation of histones changes the stability of the nucleosome or polynucleosomal structures. Although the acetylated chromatin appears slightly less condensed than control chromatin, hyperacetylation of histones does not prevent the formation of the 30-nm chromatin fiber (McGhee et al., 1983) under the solvent conditions used in these studies. However, during the nucleohistone nucleoprotamine transition, the neutralization of the positive charges of histones by hyperacetylation occurs in the presence of polycationic protamines and polyamines (Oliva & Mezquita, 1982; Oliva et al., 1982) and can produce under these circumstances disruption of chromatin structure creating sites for protamine binding (Mezquita & Teng, 1977b; Oliva & Mezquita, 1982).

If proteolysis of histones occurs at the end of spermiogenesis, as has been suggested (Marushige & Marushige, 1983), proteases would hydrolyze histones after their displacement from DNA by the protamine. Proteolysis of histones not complexed with DNA has been observed in other systems (Tsurugi & Ogata, 1979; Tsurugi et al., 1983).

Registry No. Salmine A_1, 68822-53-7; galline, 61164-22-5.

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