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Modification of Histone Binding in Calf Thymus Chromatin by Protamine†

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ABSTRACT: When calf thymus chromatin is incubated with protamine, the protein binds to DNA, forming a chromatin–protamine complex. The binding reaches a saturating level at the weight ratio of protamine to DNA of approximately 0.5. Although the saturated binding of protamine to DNA does not cause major displacement of histones from calf thymus chromatin, examination of the dissociation profiles by salt in combination with urea of protamine-treated chromatin shows that the histone–DNA interactions are markedly altered by such binding. The dissociation of histones from the chromatin–protamine complex requires less NaCl but the same concentration of urea as that for untreated chromatin, suggesting that the electrostatic interactions between the histones and DNA are decreased as a result of protamine binding. When protamine concentration is increased beyond that required for saturated binding to DNA during *in vitro* exposure of calf thymus chromatin to protamine, lysine-rich histone is completely displaced.

Chromosomal DNA becomes tightly packaged during transformation of spermatids into spermatozoa at the terminal stages of spermatogenesis. In the salmonid and related fish, the packaging of DNA occurs as a result of replacement of the entire complement of somatic-type histones by protamine (Alfert, 1956; Ingles *et al.*, 1966; Marushige and Dixon, 1969). The mechanism by which somatic-type histones are totally displaced has not been fully understood. Histones isolated from nucleohistone portions of trout spermatid chromatin show chromatographic profiles and amino acid compositions indistinguishable from those of calf thymus chromatin (Marushige and Dixon, 1971). Further-

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more, both the order of dissociation and the salt concentrations required for dissociation of histone fractions of spermatid chromatin are essentially identical with those for calf thymus chromatin (Marushige and Dixon, 1971). Therefore, there appears to be no major change either in the types of histones or in their bindings to DNA before spermatids enter the replacement stage. Histone fractions obtained from trout spermatid chromatin undergoing active chromosomal transformation have been found to be highly enriched with lysine-rich histone (Marushige and Dixon, 1969,1971). This histone fraction appears, therefore, to be the last one to be displaced during the process of replacement. A possibility that protamine might act as a dissociating agent for histones has been tested by treating nucleohistone *in vitro* with protamine. Evans *et al.* (1970) and Marushige and Dixon (1971) have found that, in contrast to the displacement of histones *in vivo,* the lysine-rich histone is the first and only major histone to be completely dissociated upon *in vitro* exposure of nucleohistone to varying concentrations of protamine. This difference clearly indicates that although the highly basic protamine is likely to play a key role in the displacement process, removal of histones during the course of replacement cannot be explained simply by direct displacement by protamine. As a step toward elucidating the mechanism of the complete change of chromosomal basic proteins during maturation of spermatids, the present study deals with the binding of protamine to somatic chromatin and with the modification of histone–DNA interactions as a result of protamine binding.

Materials and Methods

Preparation of Calf Thymus Chromatin and Salmon Nucleoprotamine. Calf thymus chromatin was prepared according to the method of Marushige and Bonner (1966), with a modification in the washing steps. Frozen calf thymus (Pel-Freez Biologicals) was homogenized in saline–EDTA (0.075 M NaCl–0.024 M EDTA (pH 8)), and the homogenate was centrifuged at 1500*g* for 10 min as originally described. The sediment was successively washed twice with saline–EDTA containing 0.5% Triton X-100 and 0.01 M Tris buffer (pH 8), and once with 0.01 M Tris buffer (pH 8) by resuspension and centrifugation (1500*g,* 10 min). The sediment was next homogenized in 0.01 M Tris buffer (pH 8) with a Teflon homogenizer by hand strokes and centrifuged at 10,000*g* for 10 min. This step was repeated once. The final gelatinous sediment was resuspended in the Tris buffer. The chromatin suspension was then mixed well with an equal volume of glycerol and stored at −20° until use. For each set of experiments, an appropriate amount of the chromatin in 50% glycerol was diluted five times with 0.01 M Tris buffer (pH 8) and centrifuged at 10,000*g* for 10 min. The sediment was suspended in the Tris buffer at a concentration equivalent to approximately 2 mg of DNA/ml and was then sheared in a VirTis homogenizer at 50 V for 3 min. Most of calf thymus chromatin was solubilized by the shearing, and was recovered as the supernatant after centrifugation at 17,000*g* for 20 min.

Salmon nucleoprotamine was prepared from salmon sperm nuclei (Sigma) in the same manner as described for preparation of calf thymus chromatin, except that the nucleoprotamine was obtained as the insoluble sediment after shearing and centrifugation (Marushige and Dixon, 1969).

Purification of Protamine. Protamine sulfate (Schwarz/Mann) was dissolved in 0.2 M HCl and the insoluble materials were removed by centrifugation (17,000*g,* 20 min). Protamine was then precipitated from the acid extract with 20% trichloroacetic acid. The precipitate was collected by centrifugation at 17,000*g* for 20 min, and washed once with acidified acetone (0.1 ml of concentrated HCl in 200 ml of acetone) and twice with acetone, and dried *in vacuo.* Protamine was next dissolved in 0.2 M acetic acid and purified by chromatography on a Bio-Gel P-10 column (2 × 20 cm) according to Ingles and Dixon (1967).

Treatment of Chromatin with Protamine. Calf thymus chromatin was treated with various concentrations of protamine in 0.01 M Tris buffer (pH 8). The concentration of chromatin was equivalent to either 300 or 600 μg of DNA/ml. While the chromatin solution was being vigorously mixed, protamine was added and this mixture was kept in an ice bath for 40 min. The mixture was then centrifuged at 17,000*g* for 20 min. The supernatant was acidified with 4 M HCl to a final concentration of 0.2 M, dialyzed against 0.87 M acetic acid overnight, and analyzed by gel electrophoresis. The sediment, from which essentially all the DNA was recovered, was treated (0°, 30 min) with 1 ml of 0.2 M HCl and centrifuged at 17,000*g* for 20 min. Acid-soluble proteins were then dialyzed against 0.87 M acetic acid for electrophoretic analyses while the acid-insoluble materials were analyzed for DNA.

Dissociation of Chromatin by Salt. Calf thymus chromatin at the concentration equivalent to 300 μg of DNA/ml was first treated with protamine at 100, 150, or 300 μg/ml in a total volume of 2 ml, as described above. At each protamine concentration the treatment was made in four identical tubes. After the treatment, the chromatin was sedimented at 17,000*g* for 20 min, and resuspended in a mixture containing 0.01 M Tris buffer (pH 8), 7 M urea, 0.1 M 2-mercaptoethanol, and either 0, 0.1, 0.3, or 0.6 M NaCl. As controls, calf thymus chromatin and salmon nucleoprotamine were appropriately diluted to a final concentration equivalent to 100–120 μg of DNA/ml with the identical mixtures as mentioned above containing the various concentrations of NaCl. The mixtures were kept at 4° for 24 hr and then centrifuged at 102,000*g* for 18 hr in a Spinco Type 65 rotor. After centrifugation the upper two-thirds of the supernatant was pipetted off, dialyzed overnight against 0.87 M acetic acid, and analyzed by gel electrophoresis. The sediment was treated (0°, 30 min) with 2 ml of 0.2 M HCl and acid-soluble proteins were electrophoretically analyzed after dialysis against 0.87 M acetic acid. Acid-insoluble materials were used for determination of DNA.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gels (5 mm × 100 mm) containing 6 M urea were prepared as described by Panyim and Chalkley (1969). Protein samples in 0.01–0.1 ml in 0.87 M acetic acid–20% sucrose were applied to the gels and were electrophoresed using 0.87 M acetic acid as the electrode buffer at 150 V for 170 min. The gels were stained overnight in 0.1% Buffalo Black containing 20% ethanol–7% acetic acid and destained electrophoretically sideways in 10% ethanol–7% acetic acid.

Chemical Analyses. DNA was determined spectrophotometrically after hydrolysis in 5% perchloric acid (100°, 10 min), using an $A_{260\,nm}$ of 1 mg/ml of hydrolyzed DNA equal to 28. Protamine was determined by the method of Lowry *et al.* (1951) standardized with protamine which had been previously quantitated by arginine determination according to Satake and Luck (1958).

Results

When calf thymus chromatin solubilized by shearing is

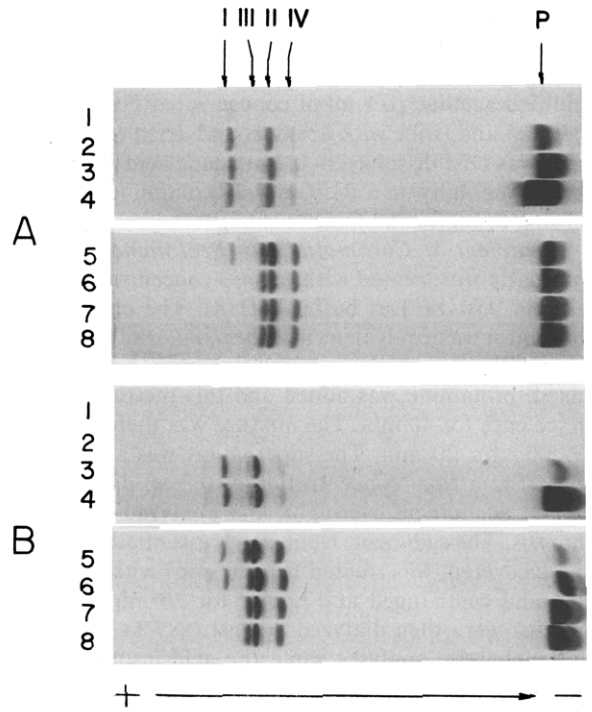

FIGURE 1: Treatment of calf thymus chromatin with protamine. Calf thymus chromatin, at the concentration equivalent to 300 (A) or 600 (B) μg of DNA/ml, was treated with protamine at 150 (1 and 5), 300 (2 and 6), 500 (3 and 7), and 1000 (4 and 8) μg/ml. Acid-soluble proteins displaced from (1–4) or remaining bound to (5–8) the chromatin equivalent to 25 μg of DNA were then analyzed by gel electrophoreses.

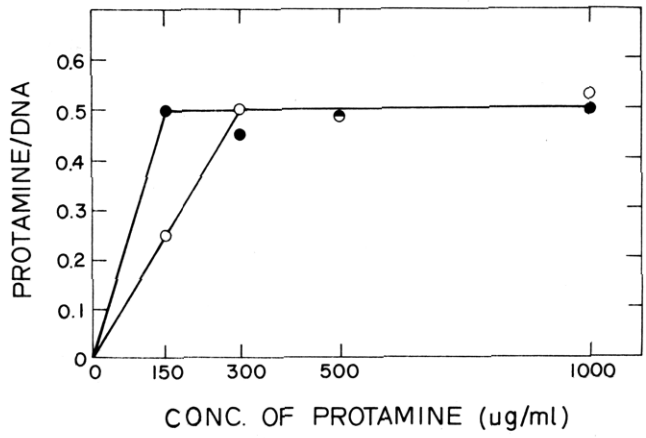

FIGURE 2: Binding of protamine to calf thymus chromatin. Calf thymus chromatin, at the concentration equivalent to 300 μg (●) or 600 μg (○) of DNA/ml, was treated with protamine at 150, 300, 500, and 1000 μg/ml. Calf thymus chromatin, thus treated, was sedimented at 17,000*g* for 20 min and the amount of protamine relative to DNA by weight was determined. At lower concentrations of protamine (150 μg/ml at the chromatin concentration equivalent to 300 μg of DNA/ml, and 150 μg/ml and 300 μg/ml at the chromatin concentration equivalent to 600 μg of DNA/ml) where all the added protamine binds to the chromatin (*cf.* Figure 1), the input ratios of protamine to DNA were plotted in the figure. At higher concentrations of protamine where histone I is completely removed from the chromatin (*cf.* Figure 1), protamine was precipitated from the acid extracts with 20% trichloroacetic acid after removing histone II, III, and IV by 5% trichloroacetic acid precipitation. The protamine was then determined according to Lowry *et al.* (1951) as described in the Materials and Methods section.

treated with protamine, the chromatin aggregates and becomes sedimentable at 17,000*g* (20 min). After treating calf thymus chromatin with various concentrations of protamine, both the acid-soluble proteins displaced from and those remaining bound to the chromatin have been analyzed by gel electrophoresis. At a chromatin concentration equivalent to 300 μg of DNA/ml (Figure 1A) lysine-rich histone I is completely displaced at a protamine concentration of 300 μg/ml (Figure 1A, 2 and 6). At this protamine concentration substantial dissociation of slightly lysine-rich histone II is also observed. Most of the arginine-rich histones, III and IV, remain associated with the DNA even at the highest protamine concentration tested (1 mg/ml). When the chromatin concentration is doubled (Figure 1B), protamine concentration required for histone displacement increases. Complete removal of histone I now occurs at 500 μg/ml. The general pattern of *in vitro* displacement of histones of calf thymus chromatin by protamine found in this study is in agreement with previous results obtained upon *in vitro* exposure of chicken erythrocyte chromatin (Evans *et al.*, 1970) and trout spermatid nucleohistone (Marushige and Dixon, 1971) to protamine.

As seen in Figure 1 (A, 1 and 5; B, 1 and 5; B, 2 and 6), all of the added protamine becomes associated with the chromatin at input ratio of protamine to DNA by weight of 0.5 or lower. When the input ratio is increased beyond 0.5, increasingly greater amounts of the added protamine remain unbound (Figure 1A, 2–4; Figure 1B, 3 and 4). Determination of weight ratios of protamine to DNA in calf thymus chromatin treated with various concentrations of protamine (Figure 2) shows that protamine binds stoichiometrically to the chromatin, and that the binding becomes saturated when the ratio of protamine to DNA reaches approximately 0.5. Native salmon nucleoprotamine prepared in the present experiment has been found to possess a similar ratio of protamine to DNA (0.53) which, in turn, agrees well with the value of 0.54 found in rainbow trout nucleoprotamine (Marushige and Dixon, 1969). When the binding of protamine to chromatin (Figure 2) is compared with the displacement of histones from chromatin (Figure 1), it is observed that only a small amount of histone is dissociated at the point where protamine binding to chromatin just becomes saturated. Dissociation of histones from chromatin occurs in the presence of excess polycationic protamine in the incubation medium.

Li *et al.* (1973) have estimated that in calf thymus chromatin approximately 80% of the DNA–phosphates are neutralized by basic residues of histones. As shown in Figure 2, calf thymus chromatin saturated with protamine possesses the weight ratio of protamine to DNA of 0.5. Since this amount of protamine is enough to neutralize as much as 75% of the DNA–phosphates on the basis of its arginine content, it seems likely that the binding of histones in calf thymus chromatin is altered from its original state as a result of the binding of protamine to the chromatin. To examine this possibility, dissociation of calf thymus chromatin–protamine complexes has next been investigated using NaCl in combination with urea. Urea is known to have a profound effect on dissociation by salt of histones II, III, and IV from isolated chromatin, whereas dissociation of histone I is little affected (Bartley and Chalkley, 1972; Kleiman and Huang, 1972). In the dissociation of chromatin by salt alone, histones dissociate in the order of I, II, and III and IV (Ohlenbusch *et al.*, 1967; Fambrough and Bonner, 1968; Wilhelm and Champagne, 1969; Marushige and Dixon, 1971). In the presence of a sufficiently high concentration of urea (*e.g.*, 7 M), dissociation of histones II, III, and IV occurs at a much lower concentration of NaCl than that required in its absence (Kleiman and Huang, 1972). Present data show that

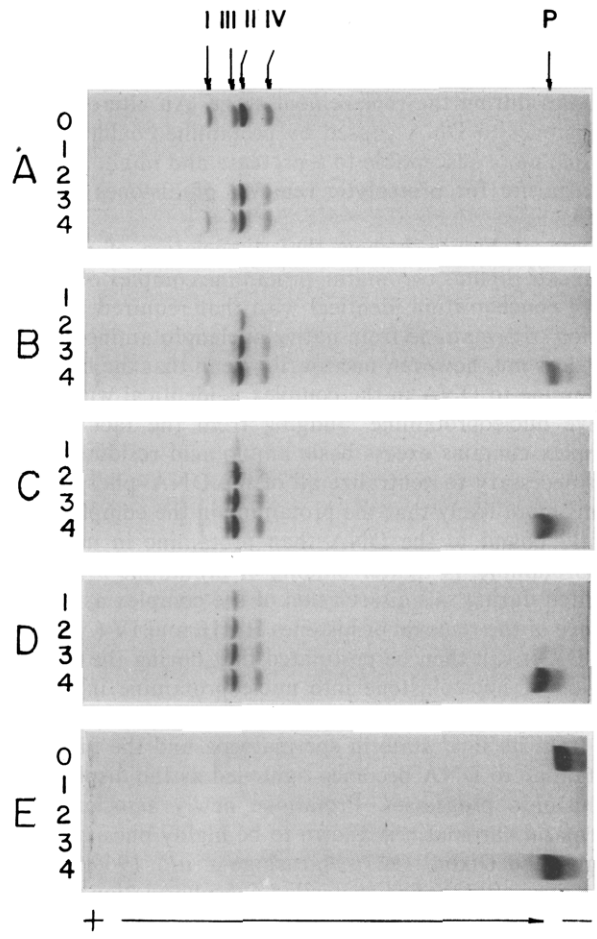

FIGURE 3: Salt dissociation of calf thymus chromatin, calf thymus chromatin-protamine complex, and salmon nucleoprotamine in the presence of urea. Calf thymus chromatin (A), calf thymus chromatin treated with 100 μg/ml (B), 150 μg/ml (C), and 300 μg/ml (D) of protamine, and salmon nucleoprotamine (E) were incubated at various concentrations of NaCl in the presence of 7 M urea. Acid-soluble proteins remaining bound to DNA in calf thymus chromatin (A-0) and salmon nucleoprotamine (E-0) after their treatment with 7 M urea alone, and those dissociated at 0 (1), 0.1 (2), 0.3 (3), and 0.6 M (4) NaCl were analyzed by gel electrophoresis. Protein samples from the control chromatins and the various chromatin-protamine complexes equivalent to 15 μg of DNA were applied to the gels.

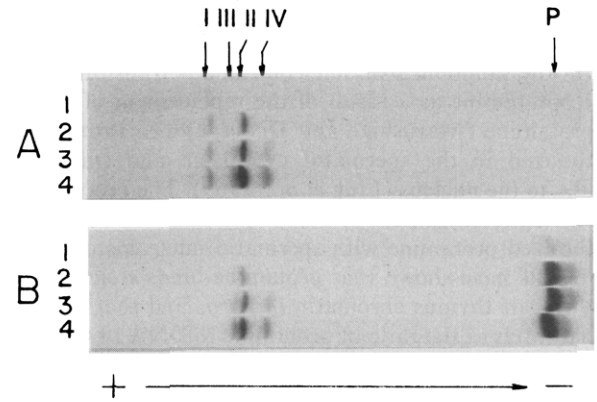

FIGURE 4: Effect of urea on salt dissociation of calf thymus chromatin and calf thymus chromatin-protamine complex. Untreated calf thymus chromatin and calf thymus chromatin treated with 300 μg of protamine/ml were incubated in 0.6 M NaCl-0.01 M Tris buffer (pH 8)-0.04 M $NaHSO_3$-0.1 M 2-mercaptoethanol containing 0 (1), 2 (2), 4 (3), and 6 M (4) urea at 4° for 5 hr. The mixtures were then centrifuged at 102,000*g* for 18 hr. The upper two-thirds of the supernatant was pipetted off, and dialyzed against 0.87 M acetic acid. Acid-soluble proteins dissociated from the chromatin equivalent to 15 μg of DNA were analyzed by gel electrophoresis.

in calf thymus chromatin (untreated control), histones II, III, and IV dissociate first at 0.3 M NaCl (Figure 3A, 3) and then histone I at 0.6 M NaCl (Figure 3A, 4). Neither 7 M urea alone nor 0.1 M NaCl-7 M urea causes appreciable dissociation of histones from calf thymus chromatin (Figure 3A, 0, 1, and 2). Figure 3B-D represents profiles of histone dissociation by NaCl-urea from calf thymus chromatin which has been pretreated with various concentrations (100-300 μg/ml) of protamine at the chromatin concentration equivalent to 300 μg of DNA/ml. In calf thymus chromatin treated with 100 μg/ml of protamine (Figure 3B), a substantial amount of histone II is removed from DNA at 0.1 M NaCl (Figure 3B, 2). When the protamine concentration is raised to 150 μg/ml (Figure 3C), considerable dissociation of histones III and IV occurs at 0.1 M NaCl (Figure 3C, 2) and some of the histone II now dissociates with 7 M urea alone (Figure 3C, 1). Also, a significantly larger proportion of histone I is removed at 0.3 M NaCl (Figure 3C, 3 and 4) than at this same NaCl concentration in the control chromatin (Figure 3A) or in the chromatin which has been treated with 100 μg/ml of protamine (Figure 3B). In calf thymus chromatin treated with 300 μg/ml of protamine (Figure 3D) dissociation profiles of histones III and IV are similar to those in calf thymus chromatin treated with 150 μg/ml of protamine. However, at the higher protamine concentration more histone II is dissociated with 7 M urea alone. As seen in Figure 3 (B, 4; C, 4; and D, 4) protamine of the calf thymus chromatin-protamine complexes dissociates at 0.6 M NaCl as does protamine of salmon nucleoprotamine (Figure 3E, 4).

Requirement of urea for salt dissociation of histones II, III, and IV is next compared between calf thymus chromatin and calf thymus chromatin-protamine complex. In Figure 4 calf thymus chromatin at the concentration equivalent to 300 μg of DNA/ml has been treated with 300 μg/ml of protamine. In this complex histone I whose dissociation is little affected by urea, is absent (*cf.* Figure 1A). The untreated calf thymus chromatin, as the control (Figure 4A), and the chromatin-protamine complex (Figure 4B) have been treated with various concentrations of urea in the presence of 0.6 M NaCl, and the acid-soluble proteins dissociated have been analyzed by gel electrophoresis. There appears to be no major change in the urea requirement as a consequence of the binding of protamine to calf thymus chromatin. Histone II of both the control chromatin and the chromatin-protamine complex dissociates at 2-4 M urea in the presence of 0.6 M NaCl, while a higher concentration of urea (4-6 M) is required for dissociation of histones III and IV. It is observed that only a small amount of protamine is dissociated with 0.6 M NaCl alone (Figure 4B, 1) and the addition of urea enhances markedly its dissociation (Figure 4B, 2-4). As shown in Figure 3E protamine is completely dissociated from salmon nucleoprotamine at 0.6 M NaCl in the presence of urea. It has been reported that when native nucleoprotamine is treated with salt alone, a much higher concentration of NaCl (1.2 M) is required for complete removal of protamine (Marushige *et al.*, 1969). The increased ease of dissociation of protamine in the presence of urea would suggest that, as in the histone-DNA interaction in nucleohistone (Bartley and Chalkley, 1972), hydrophobic interactions may be involved in the association of protamine to DNA in nucleoprotamine.

Discussion

During the transformation of spermatids into spermatozoa in the salmonid fish, nucleohistone is transformed into nucleoprotamine as a result of the replacement of histones by protamine (Marushige and Dixon, 1969). Protamine is synthesized in the spermatid cytoplasm and transported rapidly to the nucleus (Ling *et al.*, 1969). The process of replacement is presumably initiated by association of newly synthesized protamine with spermatid nucleohistone. Present results have shown that protamine binds stoichiometrically to calf thymus chromatin *in vitro*, and that the binding saturates at the ratio of protamine to DNA by weight of 0.5 (Figure 2). The value is similar to that obtained for native salmon nucleoprotamine. Although the saturated binding of protamine to calf thymus chromatin does not cause major displacement of the histones (Figures 1 and 2), the interactions between histones and the DNA appear to be markedly altered by such binding. The decreased requirement for NaCl (Figure 3) but not for urea (Figure 4) in the dissociation of histones from the calf thymus chromatin–protamine complex when compared to untreated chromatin suggests that the electrostatic interactions of histones to the DNA in calf thymus chromatin are weakened. The altered histone binding is presumably due to partial replacement of basic amino acid residues of histones on the DNA–phosphates by the arginine residues of associated protamine. Clark and Felsenfeld (1971) have found that polylysine binds to chromatin to the extent of 0.5 lysine/nucleotide, and have suggested that 50% of the DNA of chromatin is free. It seems likely, as in the interaction of chromatin with protamine, that during treatment of chromatin with polylysine the polypeptide replaces some of the basic amino acid residues of histones on the DNA–phosphates. Therefore, the titration with polylysine would not provide an accurate measure of the amount of free DNA in chromatin. Li *et al.* (1972,1973) have shown by analyzing heat denaturation profiles that approximately 80% of DNA base pairs in calf thymus chromatin are bound by histones, and that histone binding is, in fact, altered as a result of the binding of polylysine to the chromatin.

The process of complete replacement of histones by protamine during transformation of spermatid chromatin is obviously complex and may involve a series of reactions which would cause a decreased binding of histones to DNA. The altered histone interactions with DNA as a result of protamine binding may serve as a first step toward complete removal of histone. Phosphorylation of seryl residues of all major histone fractions (Marushige *et al.*, 1969; Sung and Dixon, 1970) and acetylation of ϵ-amino groups of lysyl residues of histones II, III, and IV (Candido and Dixon, 1971,1972a,b) take place in trout testis cells during spermatogenesis. Although many of the modifications have been found to be associated with younger germ cells (Candido and Dixon, 1972c; Louie and Dixon, 1972a,b), acetylation of histones has been observed also in spermatids (Candido and Dixon, 1972c) and some of the phosphorylation appears to be associated with spermatid chromatin undergoing active chromosomal transformation (Marushige *et al.*, 1969). The question as to whether the decreased binding of histones, which occurs as a consequence of association of protamine to chromatin, in combination with modifications of histones can explain the total removal of histones from spermatid chromatin during the transformation process remains to be investigated. A highly heterogeneous series of basic protein fragments are detectable in the fraction of trout spermatid chromatin undergoing active chromosomal transformation (Marushige and Dixon, 1971), suggesting that proteolytic degradation may play a key role in removal of histones during the replacement stage. An altered binding of histones to DNA caused by protamine could make the histone more susceptible to a protease and might serve as a prerequisite for proteolytic removal of histones from the DNA.

Data of Figure 3 show that dissociation of protamine from calf thymus chromatin–protamine complex occurs at a NaCl concentration identical with that required for dissociation of protamine from native nucleoprotamine. This result does not, however, necessarily mean that the binding of protamine to DNA in the complex is identical with that in native nucleoprotamine. Judging from the fact that the complex contains excess basic amino acid residues beyond that necessary to neutralize all of the DNA–phosphates, it seems more likely that the protamine in the complex is less tightly bound to the DNA than protamine in native nucleoprotamine. A tighter binding of protamine might have resulted during salt dissociation of the complex as a consequence of the removal of histones II, III, and IV (*cf.* Figure 3B–D). It can then be postulated that during the transformation of nucleohistone into nucleoprotamine in spermatids, the initial state of protamine binding to DNA is different from its final state in spermatozoa and the binding of protamine to DNA becomes tightened as the displacement of histones progresses. Protamine newly associated with spermatid chromatin is known to be highly phosphorylated (Ingles and Dixon, 1967; Marushige *et al.*, 1969; Sanders and Dixon, 1972). The controlled phosphorylation of protamine has been suggested to play a role in its "correct" binding to DNA (Louie and Dixon, 1972a). Phosphorylation of protamine not only could control the initial binding of protamine to spermatid chromatin, but also may exert its effect during the tightening processes of protamine binding, regulating the proper packaging of the DNA.

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Interrelationships between Synthesis and Methylation of Ribosomal RNA in Isolated Novikoff Tumor Nucleoli†

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ABSTRACT: Nucleoli isolated from Novikoff hepatoma cells of the rat were previously shown to carry out synthesis of predominantly ribosomal precursor RNA and methylation of this RNA *in vitro.* In order to develop *in vitro* systems for further detailed study of these processes and their interrelationships, isolated nucleoli were incubated in a complete RNA-synthesizing medium using [5-^{3}H]cytidine 5′-triphosphate or *S*-adenosyl[*methyl*-^{3}H]methionine to measure the activities of RNA synthesis and methylation, respectively, under the same reaction conditions. Methylation of the *ribose* of the nascent ribosomal precursor RNA predominated. It occurred in close coordination with the transcriptional step by RNA polymerase as shown by the kinetic data, the analysis of labeled RNA in sucrose gradients, the inhibition by increased ionic strength or actinomycin D, and the release of labeled nucleotides by a 3′-exonuclease, venom phosphodiesterase. Methylation of the RNA *bases* occurred more slowly, continued longer after transcription ceased, and appeared to follow later in the processing of the RNA. Certain divalent cations (Mg^{2+}, Mn^{2+}, and Ca^{2+} at higher concentrations, and Zn^{2+} and Cu^{2+}) inhibited both RNA synthesis and methylation to similar extents. RNase inhibitors (bentonite and dextran sulfate) at low concentration inhibited methylation while stimulating RNA synthesis, and pyrophosphate greatly decreased RNA synthesis with relatively little effect on methylation. These results indicated that RNA polymerase and ribosomal RNA methylases can function independently despite their close relationship. An exogenous substrate for the nucleolar rRNA methylases was found: nuclear RNA prepared from Novikoff hepatoma cells, cultured in the absence of methionine, served as a good substrate for methylation of both ribose and bases. Other exogenous RNAs, including cytoplasmic ribosomal RNA from these methionine-starved cells, nucleolar RNA from normal cells, and wheat germ ribosomal RNA were almost devoid of methyl-acceptor activity. A description of these parameters helps establish isolated nucleoli as a suitable system for further study of interaction of RNA polymerase, methylases, and nucleases in control of synthesis of ribosomal RNA.

Methylation of ribosomal RNA has been previously studied by labeling intact cells with methyl-labeled methionine and by subsequently isolating fractions of nucleolar RNA (Greenberg and Penman, 1966; Wagner *et al.,* 1967; Zimmerman and Holler, 1967; Weinberg *et al.,* 1967; Muramatsu and Fujisawa, 1968). These studies indicated that incorporation of methyl groups occurred concomitantly with or very soon after the synthesis of nucleolar 45S preribosomal RNA, rather than directly into smaller intermediates or mature ribosomal RNA, and that all the methyl groups incorporated in 45S RNA were conserved in the ribosomal RNA products during the processing of this precursor. Vaughan *et al.* (1967) have demonstrated that the formation of ribosomes was completely abolished during methionine starvation of the cultured cells, although 45S RNA continued to be synthesized. Vande Woude *et al.* (1970) have indicated that the selective inhibition of ribosomal RNA methylation was the primary cause which led to the disruption of host-cell metabolism during infection by foot-and-mouth disease virus. These facts indicate that methylation of preribosomal RNA is essential for some steps during processing and maturation of ribosomes, and that ribosomal RNA methylases may be among the key factors in the regulation of cell metabolism.

We have sought to examine these critical processes in

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