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Synthesis, Acetylation, and Phosphorylation of Histone IV and Its Binding to DNA During Spermatogenesis in Trout*

(starch gels/kinetics of labeling/histone-DNA binding)

ANDREW J. LOUIE AND GORDON H. DIXON

Department of Biochemistry, The University of British Columbia, Vancouver 8, B.C., Canada

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ABSTRACT **During spermatogenesis in trout testis, histone IV is extensively modified by acetylation and phosphorylation. To examine the relationship of synthesis of histone IV to its modification, histone IV labeled with [^{3}H]aminoacids and inorganic [^{32}P]phosphate was prepared from testis cells by acid extraction and column chromatography. Purified histone IV was resolved by starch gel electrophoresis into 10 bands, of which nine are modified by acetylation and/or phosphorylation. In the first 4 hr of labeling, the diacetyl-histone IV band showed the highest proportion of [^{3}H]aminoacid label. After 12 hr of incorporation, more label was found in the triacetyl and tetraacetyl bands. A significant amount of amino-acid label in the two major bands (the unsubstituted and monoacetyl bands) of histone IV was not seen until 16 hr of incubation. From 1 to 12 days, the proportion of label in the unsubstituted and monoacetylated bands increased, while that in the tetra-, tri-, and monoacetyl bands decreased. Very little [^{3}H]aminoacid was found in the phosphorylated bands of histone IV in the first 12 hr. However, after 16 hr about 20% of the total ^{3}H was found in the phosphorylated bands. The proportion increased to 33% and remained at this level between 1 and 8 days, but, by 16 days, had decreased to 12% of the total.**

These data suggest that an "obligatory" acetylation of recently synthesized histone IV is involved in the correct binding of newly synthesized histone IV to DNA. We propose that ϵ-amino acetylation of lysyl residues 5, 8, 12, and 16 neutralizes their positive charges and allows the NH_2-terminal region of histone IV to assume the correct conformation (in this case, an α-helix), and fit into the major groove of DNA. Deacetylation then "locks" histone IV to DNA by ionic linkages. The biological significance of phosphorylation of histone IV is not known.

We have been studying the metabolism of histones and protamines (1–6) in testis obtained from both naturally maturing rainbow trout and immature male trout induced to mature by injection of salmon pituitary extracts (7, 8). In examining the relationship of histone and protamine synthesis to their phosphorylation in different cell types of trout testis (9), we were struck by the fact that a radioactive amino-acid label did not appear in the two major (i.e., unmodified and monoacetylated) bands of histone IV that were resolved from the other histones by starch gel electrophoresis, until 19 hr after the start of the incorporation. This discrepancy could not be accounted for by differential histone synthesis, since all histones are synthesized simultaneously (10). However, the starch gels used here are capable of resolving histone IV into 10 bands (4, 5), of which nine are modified by phosphorylation and/or acetylation (4, 5); thus, an alternative explanation was that the label in newly synthesized histone IV was migrating in the region of modified histone IV and did not appear in the unsubstituted band until much later. We have, therefore, devised experiments to examine the kinetics of labeling of the various modified species of histone IV to gain some insight into the possible biological significance of these modifications.

* This is paper no. II in the series: "Modifications of Histones During Spermatogenesis in Trout"; ref. 4 is paper I of this series.

MATERIALS AND METHODS

L-[^{3}H]arginine (26.4 Ci/mmol) and L-[^{3}H]lysine (3.2 Ci/mmol) were obtained from New England Nuclear; carrier-free inorganic [^{32}P]phosphate from Atomic Energy of Canada, Ltd.; penicillin and streptomycin from Baltimore Biologicals; cycloheximide and dithiothreitol from Calbiochem; Nonidet P-40 from Shell Oil; carboxymethylcellulose, phosphocellulose, and Bio-Gel P-10 from Bio-Rad; variable-thickness gel slicer from Richmond Scientific Co., Richmond, B.C., Canada; Amido Black 10B from E. Merck, Darmstadt, Germany; and NCS solubilizer from Amersham Searle.

Trout Testis. Testes at different stages of development were excised from rainbow trout in which spermatogenesis had been induced by injection of salmon pituitary extracts (7, 8). We shall call these hormonally induced trout testis.

Cell Incubations. Cell suspensions were prepared by mincing 6–8 g of tissue with a scissors in 3–4 volumes of buffer A–0.1% glucose: 50 mM Tris·HCl (pH 7.5)–5 mM $MgCl_2$–25 mM KCl–0.25 M sucrose–0.1% glucose, and gently homogenized by hand (three strokes up-and-down) in a Potter–Elvehjem homogenizer with a Teflon pestle. The cell suspension was filtered through four layers of cheesecloth.

For short-term (10 min–4 hr) incubations, the cells were centrifuged at 1000 $\times$ *g* for 10 min, and resuspended in 2.5 volumes of buffer A–0.1% glucose. The suspension (about 20 ml) was incubated for 10 min at 15–16° on a gyratory water bath before addition of radioactive precursors ([^{3}H]arginine, 100 μCi/ml; [^{3}H]lysine, 100 μCi/ml; and inorganic [^{32}P]phosphate, 300 μCi/ml final concentration), phenol red, and 100 units/ml of penicillin and streptomycin. At 10 min, 1 hr, and 4 hr after addition of radioactive label, 33% of the cell suspension was removed, and cycloheximide (0.2 mM final concentration) and 2,4-dinitrophenol (0.2 mM final concentration) were added to inhibit protein synthesis and to deplete ATP by uncoupling oxidative phosphorylation. Cells were washed with phosphate-buffered saline (pH 7.2) containing cycloheximide and 2,4-dinitrophenol to remove excess radioactive label.

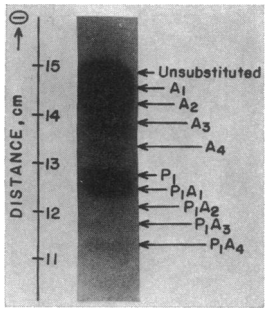

FIG. 1. A series of enzymically modified derivatives of histone IV separated by starch gel electrophoresis. Histones were extracted from hormonally induced trout testis and separated by chromatography on Bio-Gel P-10. Histone IV was well separated from other histones; the peak fractions were pooled, lyophilized, and redissolved in water. About 300 μg was applied to a starch gel slot and separated by electrophoresis at 7 V/cm for 18 hr at 6° in a water-cooled gel tray (4). Only that portion of the gel showing the protein bands is shown. The bands are labeled according to Sung and Dixon (4): the subscript numbers indicate the number of ϵ-NH_2-acetyl (A) or phosphoryl (P) groups per modified histone IV molecule.

For long-term incubations (to 12 hr), the original cell suspension (about 25 ml) was supplemented with 2.5 ml of Waymouth's medium (11) containing 10 mM Tris·HCl pH 7.2, instead of phosphate buffer, phenol red, and 100 units/ml of penicillin and streptomycin. Radioactive precursors (20 μCi/ml of [^{3}H]arginine and 200 μCi/ml of inorganic [^{32}P]phosphate) were added and the incubation was performed at 15–16° on a gyratory water bath. Control studies showed that incorporation of [^{3}H]-arginine and $^{32}P_i$ was linear for at least 12 hr under these conditions. After the incubation, the cells were diluted and washed with phosphate-buffered saline (pH 7.2).

Labeling of Testis Histones in Intact Fish. Hormonally induced rainbow trout (about 150–200 g wet weight) at the preprotamine or early protamine stage (45–55 days after the start of hormonal induction) were injected intraperitoneally with 500 μCi of [^{3}H]arginine and 500 μCi of [^{3}H]lysine in 0.4 ml of 0.15 M NaCl. At various times, fish were killed and testes cell suspensions were prepared as above.

Preparation and Fractionation of Histone. Washed cells were broken (1) in a Potter–Elvehjem homogenizer with a motor-driven pestle (5000 rpm, 30 sec). Nuclei were sedimented (2000 $\times$ g, 10 min), then resuspended in 15 ml of buffer A. 15 ml of 1% Nonidet P-40 (12) in buffer A was added to strip off any perinuclear cytoplasmic fragments, and the nuclei were sedimented again. Nuclear histones and protamines were extracted twice with 5 volumes of 0.4 N H_2SO_4 (1) and precipitated with 3 volumes of 95% ethanol. The precipitate was collected by centrifugation (15,000 $\times$ g, 10 min), washed with ethanol, and dissolved in 0.1 M LiCl. The resulting solution was titrated to pH 5.5 with 1 M Tris·HCl, pH 8, and adsorbed onto a 2.5 $\times$ 10 cm carboxymethylcellulose column (1). The column was washed with 0.2 M LiCl–10 mM lithium acetate, pH 5.5, and eluted with 0.4 M LiCl–3 M urea to remove the histones. Protamines remained on the column. The 0.4 M LiCl–3 M urea eluate was passed through a phosphocellulose column (3 $\times$ 3 cm), and washed with 0.05 M HCl. Histones were eluted with 0.4 M HCl and the eluate was lyophilized. Alternatively, total histone and protamine were eluted with 0.2 N HCl from the carboxymethylcellulose column and lyophilized. Histones were separated from protamines by passage through a 2 $\times$ 50 cm Bio-Gel P-10 column eluted with 0.01 N HCl (3). The histones were fractionated on a 3 $\times$ 320 cm Bio-Gel P-10 column as described (4, 5), except that the histones were reduced† with 20 mM dithiothreitol in 0.1 M sodium borate, pH 9.0–6 M urea, then alkylated with 40 mM iodoacetamide before application to the column (13). Fractions containing histone IV, which was clearly resolved from the other histones, were pooled and lyophilized.

Urea–Starch Gel Electrophoresis. Starch gels were prepared, and histone IV was separated by gel electrophoresis (4, 14). After electrophoresis, the gels were trisected horizontally. The bottom slab was stained by the sensitive cobalt–Amido Black 10B procedure, and destained with sulfuric acid (14). The bottom slab was further destained with 2% acetic acid for photography.

For analysis of radioactivity incorporated into the histones, the middle slab of the gel was cut into 1.5-mm slices, which were then incubated with 0.4 ml of NCS solubilizer (Amersham Searle) for 8–12 hr at room temperature (9). By the end of this period, the opaque gel had become transparent. 4 ml of toluene scintillation fluid (0.1 g POPOP–4 g PPO/liter) were then added, and the capped vials were incubated for a further 3 hr at 45° before they were counted on a Nuclear-Chicago Unilux II Counter.

RESULTS

Fig. 1 shows the separation by urea–starch gel electrophoresis of purified histone IV from hormonally induced trout testis. The 10 bands have been described (4), and result from phosphorylation of the hydroxyl group of seryl residue 1 (ref. 4) and acetylation of ϵ-amino lysyl residues at positions 5, 18, 12, and 16 (5). The bands are labeled as in ref. 4.

Fig. 2 shows the [^{3}H]aminoacid and inorganic [^{32}P]phosphate labeling patterns of histone IV from trout testis cells incubated *in vitro* with label for different lengths of time. It is apparent that in the initial 4 hr, (Fig. 2*A*, *B*, and *C*), most of the [^{3}H]aminoacid label in histone IV appears in the diacetyl derivative (A_2), with less in the mono(A_1)- and tri(A_3)-acetyl derivatives; very little radioactivity is found in the unsubstituted histone IV band (A_0). After 12 hr of labeling (Fig. 2*D*) a larger proportion of arginine label is found in A_3 and A_4 than was found in the initial 4 hr. Also, the proportion of label in A_1, one of the two major bands of histone IV (as judged by protein staining), increases significantly.

Since the enzymic modifications of histone IV occur at a relatively slow rate (Fig. 2), we examined the patterns of labeling after extended periods of time. A group of rainbow trout at a stage of development when the testis is active in histone synthesis was labeled *in vivo* by a single intraperitoneal injection of [^{3}H]arginine and [^{3}H]lysine into each fish. At various times after injection, a fish was killed, the testes were excised, and histone IV was purified.

Fig 3 shows the results of the *in vivo* labeling. After 16 hr (Fig. 3*A*), there is an appreciable proportion of [^{3}H]-aminoacid label in A_1 and A_0. In the ensuing days, the proportion of label in A_4, A_3, and A_2 decreases, while that in A_1 and A_0 increases (Fig. 3*B*, *C*, *D*, and *E*).

† Unpublished method of Dr. M. T. Sung.

In Fig. 2, very little amino-acid label was found in the phosphorylated bands of histone IV. However, 16 hr after the start of labeling (Fig. 3*A*) about 20% of the ^3H label is in phosphorylated histone IV (P_1A_0 and P_1A_1). The proportion is higher (about 25–35%) in the 1-, 3-, and 8-day samples, and gradually decreases (20% after 12 days and 14% after 16 days).

It is apparent from these experiments that very shortly after histone IV is synthesized, it is rapidly and obligatorily acetylated to (A_2). After this initial rapid acetylation, acetylation continues at a slower rate to A_3 and A_4. Acetylated histone IV is then slowly deacetylated to A_1 and A_0. The acetylation and deacetylation of a newly synthesized histone IV molecule takes about a day. These data are consistent with a model in which newly synthesized histone IV molecules pass through, in sequence, various acetylated "pools" of histone IV, and in which molecules in the acetylation pathway are recognizably different from molecules in the deacetylation pathway. It appears that the cycles of acetylation and deacetylation, and phosphorylation and dephosphorylation of histone IV take place simultaneously, but not necessarily in the same molecule at the same time. If significant phosphorylation and acetylation occurred simultaneously, on the same molecule, the ^3H-labeling patterns in the phosphorylated series of histone IV (P_1A_4 to P_1A_0) would be similar to that in the unphosphorylated series (A_4 to A_0) for labeling periods of less than 1 day (Fig. 2 and Fig. 3*A* and *B*). In fact, the two patterns are different for these early times and they suggest that phosphorylation does not occur on most histone IV molecules until they have gone through the series of acetylations to A_3 or A_4 and deacetylations to A_1 or A_0.

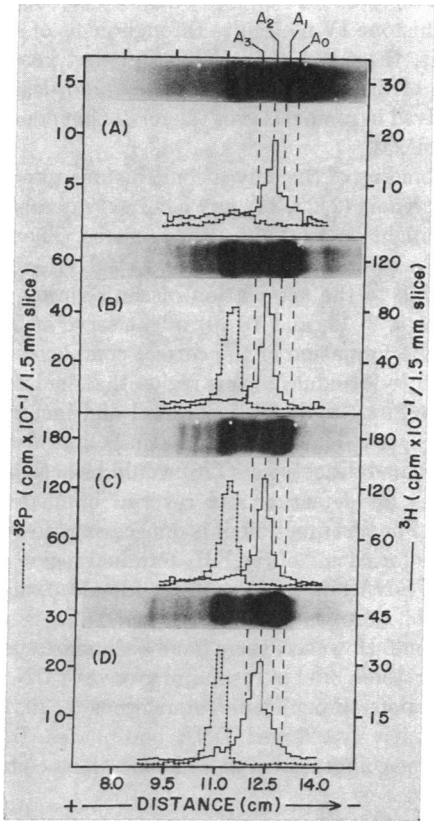

FIG. 2. Incorporation of labeled isotope into histone IV as a function of time. Trout testis cells were incubated with [^3H]-arginine and/or [^3H]lysine and inorganic [^{32}P]phosphate. At various times the incubations were stopped, histones were extracted, and histone IV was purified on Bio-Gel P-10 and resolved by starch gel electrophoresis as in Fig. 1. After electrophoresis, the gel was trisected horizontally. The bottom slab, which was stained with Amido Black 10B and destained with dilute acetic acid, is shown in the photograph. The middle slab was sliced into 1.5-mm slices, and the radioactivity of the solubilized slices was determined. (*A*), (*B*), and (*C*) are 10-min, 1-hr, and 4-hr labeling, respectively, with 100 μCi/ml of [^3H]arginine, 100 μCi/ml of [^3H]lysine, and 300 mCi/ml of [^{32}P]phosphate; (*D*), 12-hr labeling with 20 μCi/ml of [^3H]arginine and 100 μCi/ml of [^{32}P]phosphate. The blurred region slightly ahead of unsubstituted histone IV (A_0) in (*A*) is degraded histone IV. Degradation, as judged by the presence of this faster running material, sometimes occurs during repeated column chromatography or during prolonged storage at −20° of frozen aqueous samples.

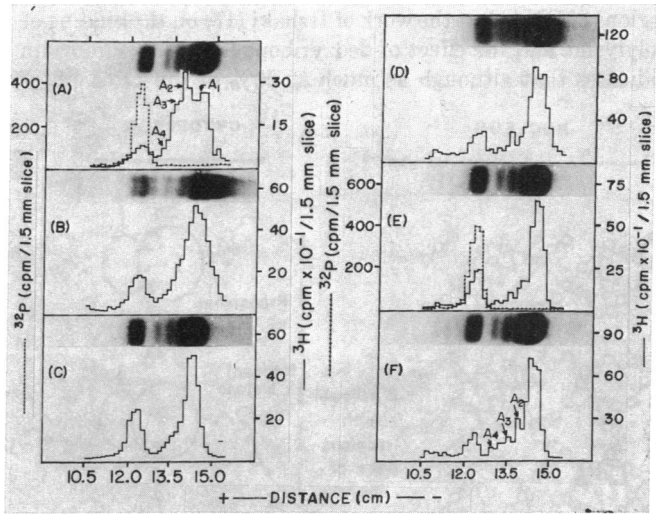

FIG. 3. *In vivo* labeling of histone IV. Rainbow trout were injected intraperitoneally with 500 μCi/ml of [^3H]arginine and 500 μCi of [^3H]lysine. At various times, a fish was killed and a cell suspension was prepared from the excised testes. In some cases, cell suspensions were also incubated for 3 hr with 300 μCi/ml of inorganic [^{32}P]phosphate to label phosphorylated histones. Histones were extracted and histone IV was separated and analyzed as in Fig. 2. (*A*), 16-hr labeling; (*B*), 1 day; (*C*), 3 days; (*D*), 8 days; (*E*), 12 days, and (*F*), 16 days.

DISCUSSION

When DNA is replicated during the cellular S phase, the total amount of histone must also be doubled. Since histone synthesis takes place in the cytoplasm (15), the question arises of how the newly synthesized histones become bound to DNA. Recent studies on trout testis cells separated by velocity sedimentation have shown that there is a positive correlation of histone synthesis and phosphorylation with DNA synthesis; cells that synthesize DNA at different rates also synthesize and phosphorylate histones at proportional rates (9). Also, studies of the cell cycle in Chinese hamster cells in culture (16) have indicated that histone acetylation occurs in S phase, during the period of histone synthesis, and that histone deacetylation takes place late in the same phase before mitosis.

We propose that those modifications that occur shortly after histone synthesis are involved in the correct binding of some of the histones to DNA in chromatin. The evidence is not strong that particular histones bind specifically to selected

regions of DNA, but the work of Itzhaki (17) on the binding of polylysine and the effect of deoxyribonucleases on chromatin indicates that although as much as 50% of the DNA phosphates are "free," few extensive tracts of free DNA exist. In addition, Marushige and Bonner (18) have shown that chromatin is not uniformly covered by histones or other chromosomal proteins. These data imply some specificity of binding of histones to DNA. In the case of histone IV, precise specificity in function of its various regions is also implied in the extreme conservation of its amino-acid sequence during evolution (19, 20).

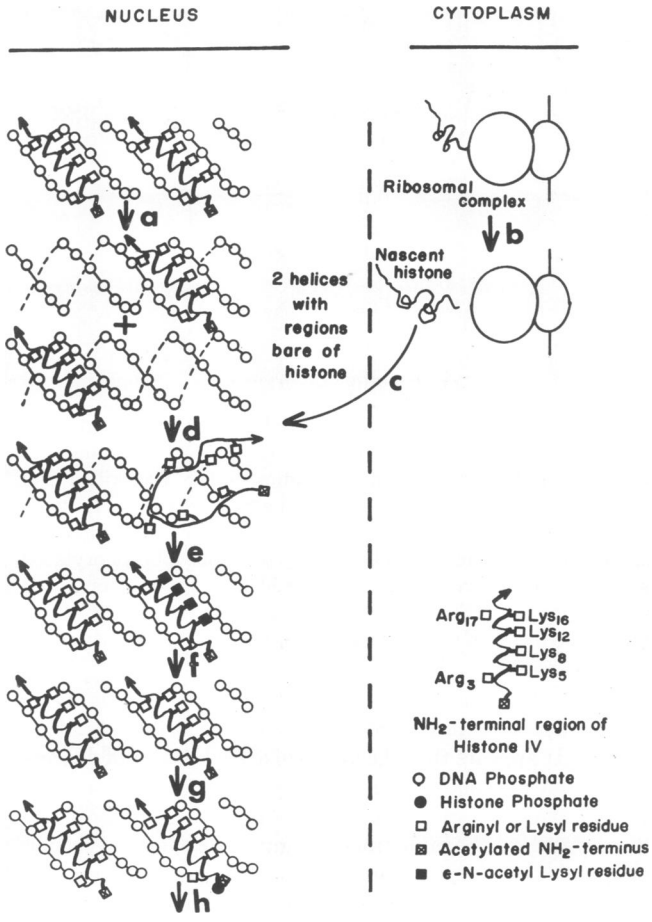

FIG. 4. A scheme for the possible binding of the basic NH_2-terminal region of newly synthesized histone IV in the major groove of DNA. For convenience, only the NH_2-terminal portion of histone IV involved with DNA binding (4, 28) is shown. How the remainder of the molecule interacts with DNA and other molecules, such as acidic chromosomal proteins and other histones, is not known. DNA synthesis (*a*) in the nucleus is accompanied by histone synthesis (*b*) in the cytoplasm. Although little is known of the partition of histones during DNA synthesis, it is assumed here that "old" histones, still correctly bound to DNA, segregate randomly (*a*) to each daughter helix. A newly synthesized histone IV chain enters (*c*) the nucleus and binds (*d*) in a random fashion through ionic linkages to DNA phosphates of the bare section of DNA. Acetylases, which act as "editing" enzymes, detect the incorrectly bound histone and acetylate (*e*) the ϵ-NH_2 groups of specific lysyl residues in the NH_2-terminal region of histone IV. This decreases their ionic interactions with the DNA phosphates and allows the neutralized NH_2-terminal region to assume the correct conformation (in this case an α-helical one) and fit into the major groove of DNA. Deacetylases, which also function as "editing" enzymes, remove (*f*) the acetyl groups to unmask the positively charged ϵ-amino-lysyl groups. This "locks" the NH_2-terminal region of histone IV in its correct DNA-binding conformation through ionic interactions with DNA phosphates. At a later stage, phosphorylation (*g*) and dephosphorylation (*h*) of histone IV occurs. The functions of phosphorylation and dephosphorylation are not known, although they may be related to the expansion and contraction of chromatin during mitosis and meiosis.

The basic regions of most of the histones are asymmetrically distributed, with clustering of basic residues and hydrophobic and acidic residues in different parts of the molecules (4, 5, 20, 21). In histone IV, the NH_2-terminal region is rich in arginyl and lysyl residues (4, 19, 20). In addition, the sites of enzymatic acetylation and phosphorylation are localized in this region (4, 5). When this evidence is combined with the observations reported here of the obligatory passage of newly synthesized histone IV molecules through pools of acetylated intermediates, there is a clear indication that postsynthetic modification of histone IV by acetylation and deacetylation may be involved in some phase of the formation of new DNA–histone complexes.

Since the binding of the polycationic histone to polyanionic DNA is very strong (2, 22, 23), any incorrect interactions once established might be very difficult to reverse. Therefore, the decrease in the positive charge density at the NH_2-terminus of histone IV due to the acetylation of the ϵ-amino groups of lysyl residues 5, 8, 12, and 16 (5) might serve an important function in the formation of the correct complex with DNA.

First, any lysyl residues in this region that had formed incorrect interactions could be acetylated and incorrect interactions eliminated. Thus, the chromatin-bound histone acetylases (24, 25) and deacetylases (26) would be acting as "editing" enzymes for detection and reversal of incorrect ionic interactions. For this function, it is unnecessary to postulate a particular conformation of the NH_2-terminal region of histone IV (or of the DNA binding sites of the histones), merely that the interaction between each histone and DNA is a definite, nonrandom one. However, there have been more specific proposals that histones bind in the major groove of DNA (27), by their basic regions through ionic interactions (4, 20, 21, 27, 28) with the negatively charged DNA phosphates. In no case, however, is the conformation of a histone, in its complex with DNA, known.

A possible model for the binding of histone IV has been proposed (4, 28) in which the first 18 residues from the NH_2-terminus are in an α-helical conformation in the major groove of DNA, and the positive charges of the four lysyl residues, 5, 8, 12, and 16, bind to a series of four phosphates on one strand of DNA. Combined acetylation of specific ϵ-amino-lysyl groups and phosphorylation of the NH_2-terminal seryl hydroxyl group in the helical portion was suggested (4) to lead to a loosening of this region, thus providing a mechanism for the modulation of histone binding to DNA and—perhaps—removal of histone during spermiogenesis, when histones are replaced by protamines (4, 22).

If the basic NH_2-terminal region of histone IV in an α-helical conformation were involved in DNA binding (4, 28), it seems likely that the negative charges of DNA would strongly stabilize this conformation once the complex had formed. However, Boublik *et al.* (29) estimated the probability of α-helix formation in various regions of histone IV in solution by arranging the known sequences according to the

helical wheel conformation of Schiffer and Edmundson (30). Using Prothero's rule (31), they came to the conclusion that the hydrophobic portion of the molecule (residues 55–72) had the highest potential for helix formation, while the most basic regions (residues 1–36 and 91–102) had the lowest, due to charge repulsion between the positively charged lysyl and arginyl residues. When histone IV was induced to become more α helical by increase of the salt concentration, high-resolution NMR spectroscopy indicated, in fact, that the prediction of little α-helix formation in the basic regions of free histone IV was justified (29). Thus, the spontaneous formation of a significant fraction of α helix in the NH_2-terminal region of free, unmodified histone IV seems unlikely.

However, the enzymatic acetylation of lysyl residues 5, 8, 12, and 16 would greatly increase the probability of α-helix formation in this region, since the main obstacle to helix formation, namely, charge repulsion of the cationic lysyl residues, would disappear. Thus, the observation in Figs. 2 and 3 that newly synthesized histone IV must pass through a series of acetylated forms is certainly consistent with the idea that these modifications are necessary to enable a particular conformation, specifically in this case an α-helix, to form before its correct binding to the major groove of DNA. Sequential deacetylation could then "lock" histone IV into place by regeneration of the positive charges of lysines 5, 8, 12, and 16 that would allow ionic interactions with four DNA phosphates. This hypothesis is outlined schematically in Fig. 4.

It takes about 1 week for stem cells of trout testis to complete the cell cycle (chromosome replication and cell division). Thus, the long period observed here for acetylation, phosphorylation, and removal of these modifying groups from histone IV is not disproportionate to the length of the trout testis cell cycle. In cells with a rapid generation time (about 1 day), such as mammalian cells in tissue culture, one would predict that the cycles of acetylation and deacetylation and of phosphorylation and dephosporylation would be considerably accelerated.

It should be mentioned that each of the histones may have different mechanisms of binding to DNA. In histone IIb_1 (also known as f2a2), the NH_2-terminal region has extensive sequence homology with histone IV (13, 32), and may also bind in an α-helical conformation to DNA (33). However, in this case, phosphorylation of histone IIb_1 begins very shortly (within 10 min) after synthesis (33), causing a decrease in the positive charge density of the NH_2-terminal region that could also help to *initiate* helix formation. This is in marked contrast to histone IV, where appreciable phosphorylation is not seen until 16 hr after synthesis (Fig. 3). In addition, in another class of chromosomal basic proteins, the protamines, we have found that postsynthetic phosphorylation also modulates their binding to DNA‡ and, hence, may affect the compactness of the chromatin. Newly synthesized protamines undergo a series of sequential phosphorylations and dephosphorylations before reaching their final unsubstituted form some 5–10 days after synthesis‡. The phosphorylation of protamine is probably involved in the correct binding of newly synthesized protamine to DNA, while the dephosphorylation of protamine is correlated with the condensation of spermatid chromatin into the very dense state characteristic of mature spermatozoa (9).

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