Processing of Newly Synthesized Histone Molecules

Nascent histone H4 chains are reversibly phosphorylated and acetylated.

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The deoxyribonucleic acid of the somatic cells of higher organisms occurs in association with small basic proteins called histones. In most cell types histones can be grouped into five major classes differing in size, positive charge, and amino acid composition. The primary structures of homologous histones from widely divergent species give evidence of remarkable evolutionary stability, particularly the arginine-rich histones, H3 and H4. Histones H2a and H2b (*1*) show slightly less evolutionary stability in their amino acid sequences, while histone H1 varies to the greatest extent (*1a*).

The structural function of histones is to organize the long, fibrillar molecules of DNA into a more compact form. This organization is achieved, in part, by electrostatic interactions between the positively charged basic amino acid residues in the histone polypeptide chains and the negatively charged phosphate groups of DNA. Current evidence favors a cooperative interaction in which four of the five major types of histones interact with each other in specific ways and in stoichiometric proportions to form multimeric protein complexes (*2*). The fundamental unit of eukaryotic chromatin may be visualized as small nucleoprotein particles (*3–5*) [called nu bodies (*4*) or PS-particles (*5*)] along the DNA molecule, in which each histone complex is enveloped by the DNA strand (*6*).

In the assembly of such particles and in their attachment to DNA, it is likely that particular regions of the histone polypeptide chains have different functions. Histone structures usually reveal a characteristic clustering of their basic amino acids—arginine, lysine, and histidine—thus generating regions of high positive charge (Fig. 1). This positively charged region is most likely to interact with the negatively charged phosphate groups of the DNA (*7*) and the less polar regions of the molecule may, in turn, interact with other histones (*2*) or with other chromosomal proteins.

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Enzymatic Modifications of Histone Structure

All nuclear histones are subject to post-synthetic modifications of structure, such as acetylation, phosphorylation, and methylation (*8*). The functional significance of such alterations in histone structure has not been definitely established, but we feel that they have considerable relevance to the organization of chromatin during DNA replication and to changes in chromatin structure at times of gene activation for RNA synthesis.

Differences in structure and organization between "active" and "inactive" chromatin can be readily visualized in various animal and plant cells. Electron microscopy of lymphocyte chromatin, for example, shows that it is distributed in two readily distinguishable forms: dense clumps of compacted fibrils, and diffuse regions of loosely extended filaments (*9*). Most of the DNA in lymphocyte nuclei, more than 80 percent of the total, is localized in the electron-opaque areas while the diffuse chromatin contains only a small fraction of the total DNA. Yet autoradiography of thin nuclear sections after labeling nuclear RNA's with [^{3}H]uridine shows that most of the radioactivity is localized in the areas of diffuse chromatin while few silver grains are present over the chromatin clumps (*9*). The extreme differences in structure and organization between "active" and "inactive" forms of lymphocyte chromatin have permitted their partial separation by physical methods to yield subfractions that differ in morphology, composition, and capacity to synthesize RNA (*10*). Although such chromatin subfractions have similar ratios of histone to DNA, they differ with regard to histone structural modifications. For example, the rate of acetylation of histones H3 and H4 is much higher in the diffuse, synthetically active chromatin fraction from calf thymus lymphocytes than in the relatively inert chromatin clumps (*11*).

Since the discovery in 1964 of histone acetylation in vivo (*12*), it has been suggested that this reaction could provide an enzymatic mechanism for modulating the interactions between histones and DNA in ways that affect the structure of chromatin (*11, 12*). This modification of the structure of DNA may, in turn, affect its template function in RNA synthesis. Support for this hypothesis is provided by numerous correlations between histone acetylation and RNA-synthetic capacity in cell types from different species and phyla—ranging from lymphocytes of the calf (*11*) to cells of the mealybug *Planococcus citrii* in which the genetically active maternal chromosomes incorporate seven times more radioactive acetate than do the inactive chromosomes of the paternal heterochromatic chromosome set (*13*). Cells of the protozoan *Tetrahymena pyriformis* also illustrate the correlation between acetylation and functional state; both the macronucleus and the micronucleus contain equivalent histone complements, but some of the histones of the active, RNA-synthesizing macronucleus occur in their acetylated forms, while histones in the inactive micronucleus are unacetylated (*14*).

Apart from such spatial correlations, there are numerous examples of increased histone acetylation at very early stages in gene activation, for example, in lymphocytes stimulated by mitogens (*15*), in hepatocytes during regeneration of the liver (*16*), and in different endocrine target cells following stimulation by the appropriate hormone (*17*). In all these cases, increased acetylation of the histones precedes an increase in RNA synthesis.

Conversely, there are correlations between the deacetylation of histones and gene inactivation. For example, in the mature sperm cells of the echinoderm *Arbacia lixula*—cells that are incapable of RNA synthesis—histones H3 and H4 occur entirely in their nonacetylated forms (*18*); these histones are partially acetylated in the synthetically active sperm-precursor cells in the testicular tissue of the same species (*19*). Similarly, there is a progressive decrease in the proportion of acetylated histone molecules in avian erythrocyte nuclei as the cells mature and lose their capacity for ribonucleic acid synthesis (*20*). In the inactivation of hepato-

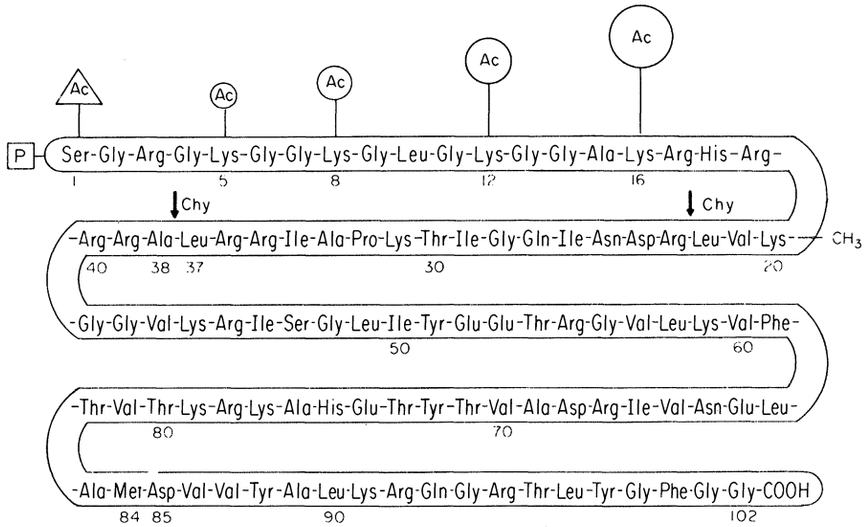


Fig. 1. Amino acid sequence of histone H4. The sites of internal acetylation are indicated by circles over the modified lysine residues. The amino-terminal serine is acetylated on its α-amino group. Phosphorylation of this serine also occurs and was detected by the recovery of [^{32}P]phosphate in the peptides released by chymotryptic cleavage (arrows) of newly synthesized ^{32}P-labeled H4 molecules.

cyte nuclei by the carcinogen aflatoxin B1, one of the earliest events is a loss of acetate from previously acetylated histone molecules; this occurs within 15 minutes after injection of the carcinogen (*21*), and it is followed by a suppression of RNA synthesis in the nuclei.

In all of the instances cited, the acetylation or deacetylation of the histones is a nuclear event (*12, 22*) that alters the structure of proteins synthesized earlier in the cytoplasm (*23*). Some clear distinctions can be made between histone modifications in the nucleus and in the cytoplasm. For example, histone H4 molecules are acetylated at their amino-terminal serine residue (*24*) (Fig. 1). This reaction takes place as an early event in histone biosynthesis when the nascent chains are still attached to small polysomes in the cytoplasm (*25*). It is essentially irreversible, and *N*-acetylserine is a permanent modification common to all histone H4 molecules. Stable amino-terminal serine acetylation is known for other histones and nonhistone proteins as well (*26*).

When histone molecules enter the nucleus they are sooner or later subject to other forms of modification. Acetylation now involves particular lysine residues within the polypeptide chain (*27, 28*). The reaction results in the neutralization of the positive charge on the ϵ-amino group of the affected lysine residue. The acetylation of lysine residues is reversible, in the sense that acetyl groups, once incorporated, are subject to enzymatic removal without degradation of the polypeptide chain (*11, 12, 15, 21, 29*).

Not all lysine residues in the histone sequence are modified in this way. The specific sites of "internal" acetylation have been identified by protein sequence studies, with the use of histones from several species. In histone H4, the sites of internal acetylation have been identified as lysine residues at positions 5, 8, 12, and 16 in the amino-terminal region of the polypeptide chain (*28, 30*) (Fig. 1). Lysine at position 16 is the major site of internal acetylation of histone H4. In histone H3, acetylated lysine residues may occur at positions 9, 14, 18, and 23 (*31, 32*). All of the sites of internal acetylation of both histones lie within highly basic regions of the molecule which are thought to interact ionically with the negatively charged phosphate-sugar-phosphate backbone of the DNA (*7, 32*). Acetylation of lysine residues in these regions would be expected to weaken ionic interactions between the histones and the DNA strand.

Individual histone molecules with identical amino acid sequences can differ with regard to their degree of acetylation. The result is that pure preparations of H3 or H4 histones are internally heterogeneous. Each histone class comprises a mixture of polypeptide chains, some of which are internally acetylated to different degrees while others are not acetylated at all. (The amino-terminal serine residue is acetylated in all H4 molecules. Because the terminal acetyl group is not subject to turnover, it is not considered a variable component in subsequent discussions of histone H4 modification.) Each of the histone subfractions may then, in addition, differ with regard to other forms of substitution, such as phosphorylation or methylation. Thus, despite the stability in amino acid sequence, the variability in histone structure is large.

Direct tests of the effects of histone acetylation on DNA structure have become possible because of the development of chromatographic and electrophoretic methods for the purification of histone subfractions that differ in their degree of acetylation (*18, 20*). Histone H4 from calf thymus nuclei has been separated into its naturally occurring nonacetylated, monoacetylated, and multiacetylated subfractions (*18*). Each of these subfractions was compared with regard to its interactions with DNA, with the use of circular dichroism to monitor changes in conformation. The results indicate that the monoacetylated form of histone H4 is far less effective than the nonacetylated form in altering the conformation of DNA, although both forms bind to the nucleic acid (*33*). This is the first direct evidence that acetylation of lysine residues in histones can alter their interactions with DNA. That this conclusion may also apply to the interactions between multimeric histone complexes and their enveloping DNA strand is strongly suggested by the fact that acetylation of histones H3 and H4 reduces the positive charge in regions of the molecule that have been found to interact with DNA in chromatin (*7*). Thus, a change in DNA conformation as a result of histone acetylation could provide a physical basis for the numerous correlations between acetylation and changes in chromatin structure and function.

Histone Structural Modifications and Chromatin Assembly

The preceding discussion has emphasized the role of histone structural modifications in the chromatin of nondividing cells, but such reactions would also be expected to play an important role in the assembly of chromatin during its replication. Here, too, it is known that DNA synthesis is most active in diffuse regions of the chromatin (*34*). In the chromosomes of some insects such as *Rhynchosciara* (*35*) or *Chironomus thummi* (*36*), sites of localized DNA synthesis are characterized by large "puffs." The implication that these regions of chromatin assembly are diffuse because histone-DNA interactions are being enzymatically modulated warrants further consideration.

It has already been suggested that multiple acetylation of the lysine residues in the most basic regions of newly synthesized histone H4 molecules, by reducing electrostatic repulsion, would allow those regions to assume an α-helical conformation and thus permit a more controlled and precise "fit" of histone H4 molecules into the wide groove of the DNA helix (*30, 32*). The model requires

multiple acetylation of each newly synthesized histone chain to ensure its correct binding to DNA. Our results on histone structural modifications in dividing erythroblasts do not support this attractive model. Instead, we have uncovered previously unsuspected, transient modifications of histone H4 in dividing cells.

The problem of histone structural modifications which accompany histone synthesis has now been reexamined, using techniques which separate histone molecules which differ only slightly in charge. The experiments to be described deal with the extent and programming of histone structural modifications in dividing erythroid cells. We have identified forms of newly synthesized histone H4 in the cytoplasm that are both acetylated and phosphorylated, and have followed the subsequent modifications of these molecules after they enter the cell nucleus.

Histone Synthesis in Dividing Erythroid Cells

The relation between biosynthesis and modification of histones has been studied in bone marrow cells from anemic Pekin ducks. Erythroblasts, both large and small, constituted about 45 percent of the marrow cell population. These dividing cells, which are known to be active in DNA (*37*) and histone (*38, 39*) synthesis, occurred together with more mature erythroid cells that had ceased replicating their DNA (*20, 37*).

Cell suspensions containing 1×10^9 to 3×10^9 cells per milliliter were incubated at 40°C with [^{3}H]lysine or [^{3}H]leucine for 1 minute to 2 hours (*40*). Structural modifications of the histones were followed by the use of [^{14}C]acetate or [^{32}P]orthophosphate (*40*).

In most experiments, the cells were labeled briefly with [^{3}H]lysine in order to identify the most recently synthesized histone molecules. Structural modifications of these molecules occurring after synthesis were observed during experiments in which the labeled cells were incubated in radioisotope-free media containing a large excess of the nonradioactive form of the precursor amino acid. Portions of the suspension were withdrawn at times ranging from 2 minutes to 12 hours, and the cells were collected, washed, and frozen at -80°C (*40*).

The erythrocyte nuclei were isolated in the presence of inhibitors of protease and phosphatase activity (*41*), and nuclear histones were extracted in 0.25*N* HCl (*42*). The histones were then separated from other proteins in the acid extract by ion-exchange chromatography on Bio-Rex 70 (*42*). This is an important step because 2 to 7 percent of the total protein in the acid extract represents a complex mixture of nonhistone proteins, some of which migrate in the positions of the histones during subsequent electrophoretic separations in acid-urea-polyacrylamide gels (*43*). These nonhistone nuclear proteins, despite their low overall concentrations, may contain up to 30 percent of the total radioactivity in the acid extract, and they must be removed. In some experiments, the purified total histone fraction was further resolved by gel filtration chromatography into components differing in molecular size (*44*). Histones in erythroblast cytoplasmic fractions were precipitated by the addition of highly polymerized exogenous DNA and then purified by chromatography on Bio-Rex 70 (*42*).

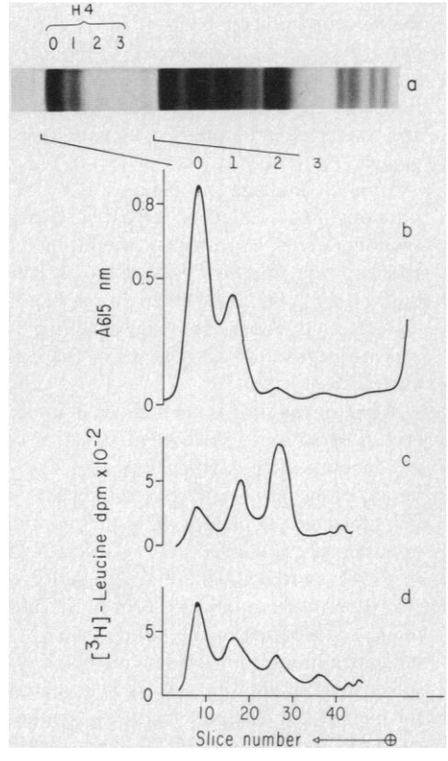


Fig. 2. Early processing of newly synthesized histone H4 molecules. The histones were labeled by incubating duck erythroid cells for 1 minute with 250 μc of [^{3}H]leucine (specific activity, 55 c/mmole). Unlabeled medium was added for 2 minutes or for 1 hour. Histones were extracted from the isolated nuclei, purified on Bio-Rex 70 columns, and separated by electrophoresis on 12 percent polyacrylamide gels (0.6 by 30 cm). (a) Distribution of histone bands in stained gels is shown. (b) The densitometric tracing of the H4 region of the gel is shown. The fast-moving unmodified H4 band is followed by slower moving bands containing H4 molecules of diminished positive charge. (c) The radioactivity profile after labeling with [^{3}H]leucine for 1 minute shows that most of the newly synthesized H4 molecules exist as modified forms of diminished mobility. (d) After 1-hour further incubation in unlabeled medium the ^{3}H-labeled H4 molecules have been processed to the predominant unmodified form. Abbreviations: dpm, disintegrations per minute; A, absorbancy.

The enzymatic modification of histone H4 by acetylation of lysine residues or phosphorylation of serine residues alters its electrophoretic mobility. The modified forms have a lower net positive charge and can be separated from the unmodified molecules by electrophoresis in polyacrylamide gels under acid conditions. We have modified the original electrophoretic method of Panyim and Chalkley (*20, 43*). The distribution of radioactivity in the various modified histone forms was determined by slicing the gels transversely and counting the radioactivity in each of the slices (*45*). After appropriate corrections for quenching and crossing-over, the radioactivity in each gel slice was plotted as a function of the distance of migration of the corresponding histone band.

When immature erythrocytes from duck bone marrow are incubated in the presence of radioactive amino acids, the isotope is rapidly incorporated into newly synthesized histone molecules. Labeled histones can be recovered from the cell nucleus after incubation for 1 minute with [^{3}H]leucine or [^{3}H]lysine, which gives some indication of the speed of peptide chain elongation (*46*) and of histone transport into the nucleus from sites of synthesis in the cytoplasm (*23*). Because the early kinetics of labeling of histones recovered from the nucleus do not show any appreciable lag phase, it follows that these cells do not contain a large pool of unlabeled cytoplasmic histones awaiting transport to the nucleus. The specific activity of the histones continues to increase for at least 10 minutes in unlabeled medium after a 1-minute exposure to isotopic amino acids, indicating continued utilization of the labeled precursors remaining in the cultured cells.

Parallel studies of DNA synthesis in the same cell population (*47*) show that [^{32}P]phosphate incorporation into DNA is linear for at least 60 minutes. Thus, by these criteria of activity, the erythroid cell culture provides a model system for the study of histone structural modifications occurring at the time of DNA replication and chromatin assembly.

Modification of Newly Synthesized Histone Molecules

When purified erythrocyte histones are separated by electrophoresis in acid-urea-polyacrylamide gels (*43*), the acetylated forms of histones H3 and H4 migrate more slowly than do the unmodified polypeptide chains, because acetylation neutralizes the positive charge of the affected lysine residues. A series of bands of progressively

lower mobility is produced as the number of acetyl groups attached to the histone molecule increases (Fig. 2, a and b).

The relation between the mobility of the histone subfractions and their acetyllysine content has been established (*18*). The leading band in the H4 region has no acetyllysine; the following bands, in order of mobility, contain one, two, three, or four acetyllysine residues, respectively. The proportions of the various forms of histone H4 in the whole marrow culture are 60 percent unacetylated, 29 percent monoacetylated, 7 percent diacetylated, and 4 percent triacetylated. Less than 1 percent occurs in the tetraacetylated form, and unless isotopic labeling procedures are used, the tetraacetylated form is barely detectable.

When histone-synthesizing cells are labeled with [^{3}H]leucine for 1 minute, the radioactivity distribution in the H4 region of the gel does not coincide with these proportions; that is, most of the newly synthesized, ^{3}H-labeled histone H4 molecules do not migrate with the quantitatively predominant, leading histone band. Instead, the leucine label is predominantly present in H4 molecules of lower mobility (Fig. 2c). However, if the 1-minute incorporation is halted by an addition of nonradioactive medium for the next hour of incubation, a shift occurs in the position of the ^{3}H-labeled H4 molecules (Fig. 2d). Most of the radioactive H4 now appears in the leading band which (except for its acetylserine terminus) is unmodified by acetylation or phosphorylation. This is a clear indication that the groups which originally altered the mobility of newly synthesized histone H4 molecules are subject to removal.

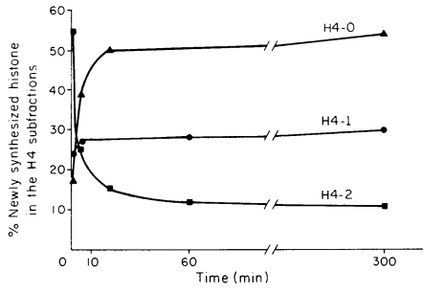


Fig. 3. Kinetics of interconversion of modified and unmodified forms of newly synthesized histone H4 molecules. Erythroid cells were labeled for 1 minute with [^{3}H]leucine and then incubated in unlabeled medium for periods up to 5 hours. The histones were prepared at intervals during the 5-hour incubation and analyzed by polyacrylamide gel electrophoresis to give the amount of ^{3}H-labeled histone in each of the H4 bands. The relative proportions of the labeled H4 subfractions, as determined by electronic integration of the radioactivity profiles, are plotted as a function of time. The singly modified form (H4-1) represents a mixture of molecules that are either phosphorylated or acetylated. The proportions of newly synthesized H4 in either form change with time. At the outset, the newly synthesized H4-1 occurs predominantly as the monophosphorylated derivative, but later it is converted to the monoacetylated derivative. These forms can be distinguished from one another by labeling with the appropriate radioisotopic precursors (Fig. 5, c and d). (▲) Percentage of newly synthesized H4 in the unmodified form (H4-0). (●) Percentage of newly synthesized H4 in the singly modified form (H4-1). (■) Percentage of newly synthesized H4 in the doubly modified form (H4-2).

The kinetics of removal of the histone-modifying groups have been studied by observing the changes in electrophoretic mobility of the newly synthesized H4 molecules at different intervals during a subsequent incubation in unlabeled medium after a 1-minute incorporation of [^{3}H]leucine. At 1 minute, more than 55 percent of the labeled H4 molecules occur in the slow-moving band (Fig. 2), while only 17 percent are recovered in the leading band. Within 20 minutes these proportions are reversed as the modified forms are converted into the fast-moving component (Fig. 3).

Similar analyses of histone H3 subfractions have failed to reveal a corresponding series of transient alterations in structure and mobility related to its synthesis. The complex electrophoretic banding pattern of histone H3 (Fig. 4a) is due to varying degrees of acetylation in the cell nucleus.

When histone H3 is synthesized in intact erythroblasts and recovered after a 1-minute labeling with [^{3}H]leucine, the distribution of the newly synthesized radioactive H3 molecules coincides with that of the preexisting, unlabeled H3 subfractions (Fig. 4c). Moreover, there are no shifts in electrophoretic mobility during a subsequent incubation in unlabeled medium to indicate a programmed attachment and release of modifying groups, as is observed for histone H4. Instead, the newly synthesized H3 molecules enter the nucleus and are acetylated in about the same proportions as are the "old" H3 molecules (Fig. 4d). Histone H3 synthesized in cytoplasmic systems (described in the legend to Fig. 6) shows only a single radioactive band appearing in the position of the unmodified H3 component. Thus, it follows that the modification of nascent chains is selective for histone H4 and is not a general phenomenon.

High-resolution gel electrophoretic techniques have permitted a closer analysis of the mobility differences between newly synthesized histone H4 molecules and the comparatively stable forms of this histone species. The spacings between the peaks of optical density in the densitometric tracings indicate the relative mobilities of histone H4 molecules which contain none, one, two, three, or four ϵ-*N*-acetyllysine residues (*18, 20*) (Fig. 5, a and c; Table 1). The areas beneath the peaks indicate the amounts of each subfraction; the relative proportions of these forms remain stable throughout the time periods considered in these experiments. However, the electrophoretic mobilities of newly synthesized H4 molecules, as indicated by the distribution of [^{3}H]lysine or [^{3}H]leucine-labeled proteins after very short labeling periods,

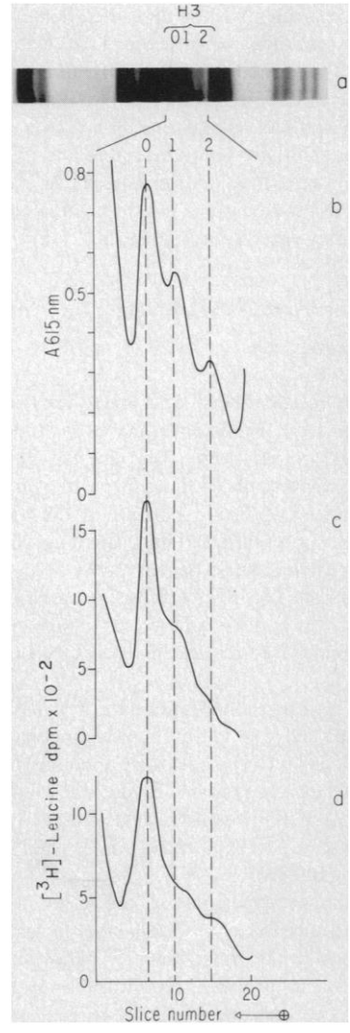


Fig. 4. Early processing of newly synthesized histone H3 molecules. The conditions of labeling cells with [^{3}H]leucine and for analysis of the purified histones are essentially as described in the legend to Fig. 2. (a) The distribution of histone bands in stained gels is shown. (b) The densitometric tracing of the H3 region of the gel is shown. The fast-moving unmodified H3 band is followed by slower-moving forms of diminished positive charge. (c) The radioactivity profile after labeling with [^{3}H]leucine for 1 minute followed by 2 minutes in unlabeled medium shows that the distribution of labeled H3 molecules resembles the distribution shown in (b) of total H3 molecules. (d) The radioactivity profile after labeling for 1 minute followed by a 5-hour incubation in unlabeled medium shows no appreciable difference in the distribution of the labeled H3 molecules.

differ from those expected for histone H4 subfractions which differ only in ϵ-*N*-acetyllysine content. The peaks of radioactivity and optical density do not match (Fig. 5, a and d, and the spacings in Table 1). It follows that some other form of structural modification has altered the mobility of the newly synthesized H4 molecules.

The nature of the additional modification has been investigated by carrying out experiments in which other isotopic precursors were used to determine the relation between electrophoretic mobility and the nature of the substituent group. The radioactivity of each of the H4 subfractions was determined after various periods of labeling.

The main points of a number of key experiments can be summarized briefly (Fig. 5):

1) Histone H4 molecules labeled with [^{3}H]lysine for 1 minute migrate differently from older H4 molecules labeled with [^{14}C]leucine for 2 hours and incubated in unlabeled medium for an additional 2 hours (Fig. 5b, upper and lower curves).

2) The positions of the older histone molecules coincide with the bands visible in the gel pattern (lower curve in Fig. 5b and tracing in Fig. 5a).

3) Histone H4 molecules labeled with [^{14}C]acetate migrate, as expected, with the densitometrically determined H4 bands, each of which corresponds to different degrees of acetylation of the parent polypeptide chain (Fig. 5c).

4) There are two classes of newly synthesized H4 molecules (detected after labeling for 1 minute with [^{3}H]lysine) which migrate more slowly than expected for the mono- and diacetylated forms of the molecule (Fig. 5c).

5) Histones labeled with [^{32}P]orthophosphate also show peaks of activity in the H4 region of the gel. Two of these coincide exactly with the positions of the newly synthesized, slowly moving forms of histone H4. There is no ^{32}P activity in the fast-moving H4 band (Fig. 5d).

Other experiments have established that the phosphorylated forms of histone H4 do not coincide in mobility with older histone molecules labeled with [^{3}H]leucine and isolated after subsequent, prolonged incubation in unlabeled medium.

Thus, newly synthesized H4 molecules occur predominantly in three forms, two of which contain phosphate and have a correspondingly lower electrophoretic mobility toward the cathode than does the unmodified histone molecule (Fig. 5d). The spacings between the unmodified and modified forms offer useful clues to the nature of the substitutions in the slow-moving bands. The band of intermediate mobility contains one phosphate group. Because of the negative charge of the phosphate group, this modified protein moves even more slowly than does the monoacetylated form of histone H4 (Table 1). It is shown later (see Appendix) that the phosphate group is esterified to the hydroxyl group of the amino-terminal serine residue. The slowest H4 component also contains [^{32}P]phosphate; but, in addition, its reduced mobility suggests the presence of an additional modification. Evidence is presented in the Appendix that an ϵ-*N*-acetyllysine residue is responsible for the further decrease in charge of the phosphorylated H4 form.

Table 1. Differences in electrophoretic mobility of histone H4 and its derivative forms.

Band separation observed by	Spacing between bands of histone subfractions		
	From	To	Distance*
Densitometry†	H4 – 0	H4 – $(Ac)_1$	1.00 (31)
	H4 – $(Ac)_1$	H4 – $(Ac)_2$	1.36 ± 0.10 (31)
	H4 – $(Ac)_2$	H4 – $(Ac)_3$	1.38 ± 0.10 (7)
Isotope distribution‡	H4 – 0	H4 – $(P)_1$	1.40 ± 0.05 (12)
	H4 – $(P)_1$	H4 – $(P)_1(Ac)_1$	1.49 ± 0.10 (12)

*The distances are expressed relative to the distance between the unmodified H4 and its monoacetylated derivative taken as 1.00. The standard deviation is indicated. Figures in parentheses give the number of determinations made. †Gels stained with amido black 10B were scanned at 615 nm, and the distances from band to band were measured using the maxima in the densitometric tracing (see Fig. 5). ‡The distance between [^{3}H]lysine-labeled (for 1 minute) histone bands was determined by sectioning the gels into 1-mm slices, each of which was analyzed for ^{3}H activity. The distances between bands were measured from the maxima of the plot of radioactivity as a function of mobility.

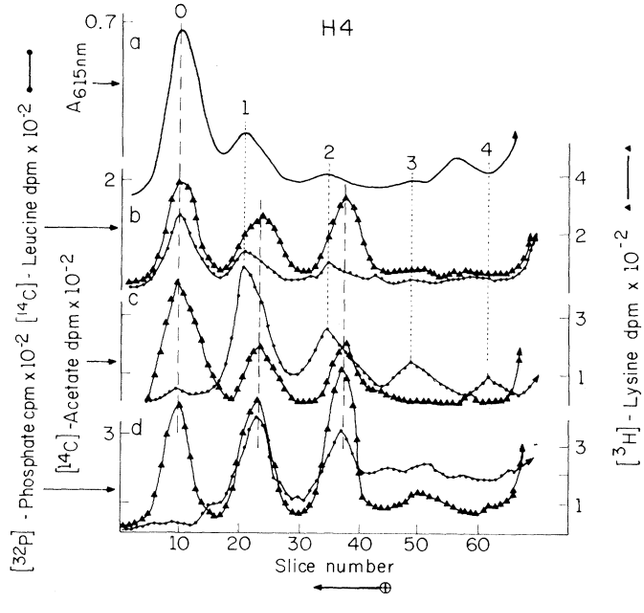


Fig. 5. Electrophoretic analysis of "new" and "old" histone H4 molecules and identification of the modifying groups affecting the mobility of the polypeptide. (a) Densitometric tracing of the histone bands in the H4 region of an 11 percent polyacrylamide gel (0.6 by 45 cm). (b) Comparison of the electrophoretic mobilities of histone H4 subfractions after short-term and long-term labeling experiments. The upper curve (▲) shows the distribution of radioactive histone molecules after labeling bone marrow cells for 1 minute with [^{3}H]lysine (300 μc/ml; specific activity, 52 c/mmole), followed by a 4-minute incubation in unlabeled medium. The lower curve (●) shows the distribution of "old" histone molecules prepared from cells incubated for 2 hours with [^{14}C]leucine (15 μc/ml; specific activity, 312 mc/mmole), followed by a 2-hour incubation in unlabeled medium. In these experiments, the ^{3}H- and ^{14}C-labeled cells were combined for isolation of the nuclei and extraction of the histones. After purification on Bio-Rex 70, the histones containing [^{3}H]lysine or [^{14}C]-leucine were analyzed electrophoretically. This procedure ensures that the different mobilities observed for the modified forms of "new" and "old" H4 molecules cannot be attributed to different experimental conditions. (c) Comparison of the electrophoretic mobilities of erythroblast H4 subfractions after short-term labeling with [^{3}H]lysine and long-term labeling with [^{14}C]acetate. The acetylation of histone H4 was carried out in erythroid cells exposed for 30 minutes to 200 μc of [^{14}C]acetate per milliliter (specific activity, 52 mc/mmole). The newly synthesized H4 molecules were labeled in the same cell population by a terminal 1-minute labeling with 200 μc of [^{3}H]lysine. After a 4-minute incubation in a medium free of [^{3}H]lysine, but containing [^{14}C]acetate, the histones were extracted, purified on Bio-Rex 70, and analyzed. The distribution of newly synthesized molecules (▲) is clearly different from that of the "older" acetylated forms of histone H4 (●). (d) Comparison of the electrophoretic mobilities of newly synthesized and phosphorylated forms of erythroblast histone H4. The cells were incubated in the presence of [^{32}P]orthophosphate (3 c/ml) for 1 hour, then the newly synthesized H4 molecules were labeled for 1 minute with [^{3}H]lysine (125 μc/ml). After a 4-minute incubation in unlabeled medium, the histones were extracted, purified on Bio-Rex 70, and analyzed. The positions of the modified forms of newly synthesized H4, as indicated by the tritium activity (▲), coincide exactly with the positions of the ^{32}P-labeled histone bands (●).

Cytoplasmic and Nuclear Processing of Newly Synthesized H4 Molecules

Several lines of evidence indicate that the phosphorylation and acetylation of newly synthesized H4 molecules take place in the cytoplasm of the erythroblast.

1) The synthesis and modification of the histones take place in a cell-free, protein-synthesizing system prepared from the postmitochondrial supernatant fraction of duck erythroblasts. The cytoplasmic system (described in detail in the legend to Fig. 6) was found to incorporate [^{3}H]lysine into protein in linear fashion for about 20 minutes, after which the rate of lysine incorporation declined. A plateau of [^{3}H]lysine uptake was reached at about 60 minutes and maintained for an additional hour. All histone classes were synthesized in this cytoplasmic system. Histones labeled with [^{3}H]lysine in vitro were recovered as histone nucleates by precipitation with exogenous DNA, with unlabeled nuclear histone as a "carrier." [Control experiments with "carrier" histones labeled with tritium by reductive methylation (*48*) showed essentially complete recovery under the conditions employed (Table 2).]

The key point is that H4 histone synthesized in the cytoplasmic system appears as a modified form, slow moving in comparison to the unmodified H4 molecules present in the added "carrier" histone fraction (Fig. 6).

The modified H4 histone is not further processed in the cell-free system as it is in vivo; it persists from 10 minutes (Fig. 6b) to labeling periods as long as 2 hours (Fig. 6c). Figure 6d shows the electrophoretic patterns of "carrier" histones before and after their recovery from the cytoplasmic fraction by precipitation with exogenous DNA. It is included to emphasize that the method allows an effective recovery of all histone fractions, including the nonmodified as well as the modified forms of histone H4. Thus, the absence of the unmodified form of H4 in the cytoplasmic histones labeled with [^{3}H]lysine is not due to methodological error. It follows that the conversion reactions leading to the unmodified form of H4 do not occur in the cytoplasmic fraction.

The newly synthesized, cytoplasmic form of histone H4 has an electrophoretic mobility corresponding to that of one of the phosphorylated forms of the molecule (Fig. 7). The [^{3}H]lysine-labeled histones prepared from the cell-free protein-synthesizing system were mixed with ^{32}P-labeled histones extracted from the nuclei of cells that had been incubated with [^{32}P]orthophosphate for 2 hours. Electrophoretic analysis of the histone mixture indicated that the ^{3}H-labeled H4 molecules made in the cytoplasmic system have the same electrophoretic mobility as the second phosphorylated form of H4 isolated from ^{32}P-labeled cells.

Histone H4 molecules synthesized in the cytoplasmic system are also acetylated (Fig. 7). This was demonstrated with [^{3}H]lysine as a precursor of the acetyllysine residues in the polypeptide chain. After 10 minutes, the histones were precipitated by the addition of highly polymerized DNA and then purified by chromatography on Bio-Rex 70. The ^{3}H-labeled histones were digested with trypsin and Pronase to release ϵ-*N*-acetyl-[^{3}H]lysine (*27*). The latter was separated from free [^{3}H]lysine and identified by chromatography and by the presence of the tritium label. (None of the other histones synthesized in the erythroblast cytoplasmic system was found to contain ϵ-*N*-acetyllysine.) Thus, the synthesis of histone H4 in the cytoplasmic system yields polypeptide chains that are both phosphorylated and acetylated. These modifications are stable in the absence of cell nuclei.

Labeling experiments in intact cells confirm this conclusion. Histone and DNA synthesis are closely coupled in a variety of cell types (*49*), and both proceed during the erythroblast stage of avian red cell differentiation (*37 39*). On the premise that a block in DNA synthesis might also affect the synthesis and processing of histone H4 and slow the utilization of newly synthesized H4 molecules for chromatin assembly, we have compared nuclear and cytoplasmic histones in erythroblasts exposed to an inhibitor of DNA synthesis, hydroxyurea. The inhibitor was used at a concentration (2 m*M*) that inhibits

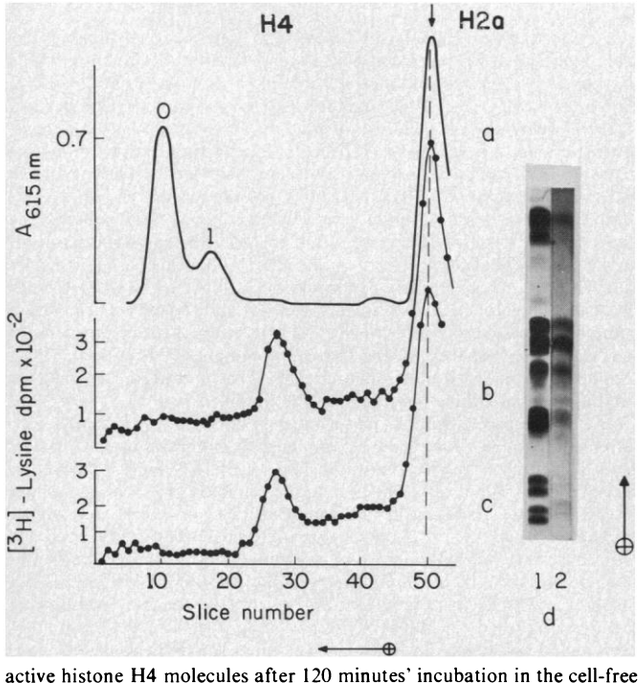


Fig. 6. Cytoplasmic synthesis and modification of histone H4. A cell-free protein-synthesizing system was prepared from bone marrow cells that had been incubated in a lysine-free medium for 40 minutes at 40°C. The cells were washed at 4°C in a solution of 130 m*M* NaCl, 5.2 m*M* KCl, and 7.5 m*M* $MgCl_2$ and lysed in one-half volume of 2 m*M* $MgCl_2$. After 3 minutes, one-half volume of a solution consisting of 400 m*M* KCl, 12 m*M* $MgCl_2$, 4 m*M* dithiothreitol, and 20 m*M* tris-HCl, *p*H 7.4, was added; the lysate was centrifuged at 16,000*g* for 10 minutes. The supernatant was made 1 m*M* in adenosine triphosphate (ATP), 0.2 m*M* in guanosine triphosphate (GTP), 15 m*M* in creatine phosphate, and 136 μg of creatine phosphokinase was added. The system was brought to 40°C; 100 μc of [^{3}H]lysine (specific activity, 50 c/mmole) was added. Samples were taken at different times and chilled before adding 2 mg of nonradioactive duck erythrocyte whole histone as a "carrier." The histones were then precipitated as histone nucleates by the addition of highly polymerized DNA (Table 2). After the precipitate was washed with 0.14*M* NaCl, the histones were recovered from the DNA-histone complex by overnight extraction in a mixture of 6*M* urea, 4*M* guanidine hydrochloride, and 10 m*M* β-mercaptoethanol in 50 m*M* tris-HCl, *p*H 7.4. The DNA was sedimented by centrifugation in a SW 65 Ti rotor at 64,000 rev/min for 14 hours. The histones in the supernatant were dialyzed against a solution of 0.36*M* NaCl, 40 m*M* NaF, 1 m*M* $NaHSO_3$, and 10 m*M* tris-HCl, *p*H 7.0. Small precipitates formed during dialysis were extracted with 0.25*N* HCl to recover the lost histone, and the extract was dialyzed as before. The combined extracts were then purified on Bio-Rex 70 before electrophoretic analysis. (a) Densitometric tracing of the H4 region of the gel. (b) Distribution of radioactive histone H4 molecules after 10 minutes' labeling in the cell-free protein-synthesizing system. Note the retarded mobility of the newly synthesized H4 chains. (c) Distribution of the radioactive histone H4 molecules after 120 minutes' incubation in the cell-free system. Note the absence of any cytoplasmic conversion of the slow-moving form of histone H4 to the fast-moving unmodified H4 band. (d) Electrophoretic analyses of the "carrier" histones (gel 1) and of histones recovered from the cell-free system (gel 2) by precipitation with exogenous DNA. The banding patterns of the erythrocyte histones recovered from the DNA complex indicate the presence of all histone fractions including the nonmodified as well as the modified forms of histone H4.

[^{32}P]orthophosphate incorporation into DNA by 60 percent. Under these conditions, cytoplasmic histone H4 accumulates in the modified form (Fig. 8, lower curve) while the nuclear H4 shows a partial conversion to the unmodified form (Fig. 8, upper curve).

Nuclear Processing of Newly Synthesized Histone H4 Molecules

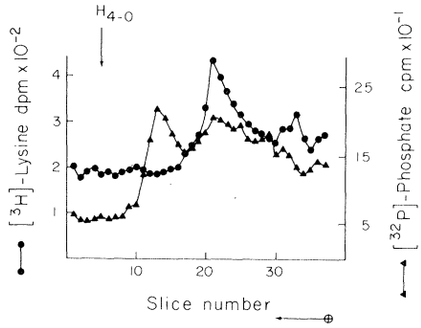
Intact cells rapidly convert the newly synthesized, slow-moving forms of histone H4 into the fast-moving form that predominates in the cell nucleus (Fig. 2). The cytoplasmic histone-synthesizing system does not further process the H4 molecules containing serine-phosphate and ϵ-*N*-acetyllysine (Fig. 6). It follows that the dephosphorylation of the serine residue and the deacetylation of the lysine residue are nuclear events which rapidly alter the structure of histone H4 molecules entering from the cytoplasm.

This conclusion has been confirmed by the identification of the original, intermediate, and final forms of newly synthesized H4 molecules in isolated erythroblast nuclei. In order to rule out artifacts of translocations of histones between cytoplasm and nucleus during the course of nuclear isolation, the cells were immediately frozen in Freon-12 at −156°C and lyophilized prior to isolation of the nuclei in nonaqueous media (*50*).

Radioactive histones isolated from the nucleus after a 1-minute "labeling" with [^{3}H]leucine show a predominance of the newly synthesized "cytoplasmic" form I of histone H4 (Figs. 9b and 11). Also present are an intermediate form II, which is phosphorylated but not acetylated, and the final product of "early nuclear processing," an H4 form III lacking both acetyllysine and phosphoserine (Fig. 9b).

The electrophoretic pattern of old histones prepared from nonaqueous nuclei that were labeled in vivo with [^{3}H]leucine and then subjected to a 1-hour incubation in unlabeled medium is very different from that obtained immediately after the 1-minute exposure to ^{3}H. The distribution of the radioactive histones in the gel now closely parallels the densitometric tracing of the stained bands corresponding to the unmodified H4 and its acetylated derivatives (Fig. 9, a and c).

It can be concluded that later stages in the nuclear processing of histone H4 involve a series of acetylation reactions along the polypeptide chain, each reaction giving rise to an additional ϵ-*N*-acetyllysine residue and leading to a stepwise re-



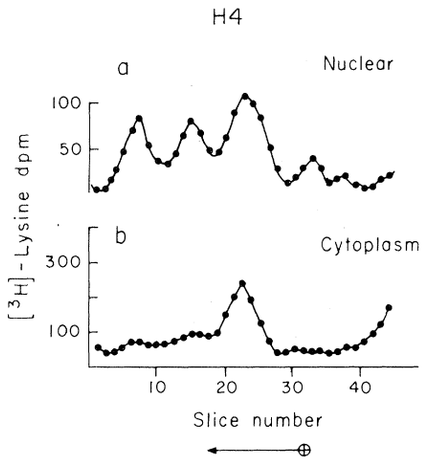


Fig. 7 (left). Comigration of phosphorylated histone H4 molecules with the newly synthesized H4 molecules from a cytoplasmic protein-synthesizing system. Histones labeled with [^{32}P]phosphate were prepared from immature erythroid cells which were labeled for 2 hours with [^{32}P]orthophosphate prior to isolation of the nuclei and purification of the histones in the acid extract by Bio-Rex 70 chromatography. Histones thus obtained were used as "carriers" for the precipitation of [^{3}H]lysine-labeled histones prepared from the erythroblast cytoplasmic protein-synthesizing system described in the legend to Fig. 6. After 60 minutes' incubation with [^{3}H]lysine, the newly synthesized cytoplasmic histones (plus the added "carrier" histones) were precipitated as histone nucleates. Electrophoretic analysis of the doubly labeled histone mixture shows that the [^{3}H]lysine-containing H4 molecules synthesized in the cytoplasmic system (●) comigrate with the second phosphorylated H4 component prepared from the nuclei of the ^{32}P-labeled cells (▲). Fig. 8 (right). Accumulation of modified histone H4 molecules in the cytoplasm of erythroid cells when DNA synthesis is inhibited. Bone marrow cells were incubated in the presence of [^{32}P]orthophosphate to monitor DNA synthesis in the presence and absence of 2 m*M* hydroxyurea. Under conditions in which DNA synthesis was inhibited by 60 percent, the erythroblast histones were labeled by a 15-minute incubation with [^{3}H]lysine (80 μc/ml; specific activity, 52 c/mmole). The cells were lysed, homogenized in the presence of sonicated DNA (Table 2), and fractionated to yield nuclear and cytoplasmic fractions. Histones were extracted from both sources, purified on Bio-Rex 70, and analyzed on 11 percent polyacrylamide gels. (a) Radioactivity profile of the H4 histones from isolated nuclei. Both modified and unmodified forms of the newly synthesized H4 molecules are present. (b) Radioactivity profile of the cytoplasmic histone H4 recovered by DNA precipitation (legend to Fig. 6). The slow-moving modified form of H4 is predominant.

Table 2. Recovery of radioactive histones as histone nucleates. Abbreviation: dpm, disintegrations per minute.

Procedure	Total ^{3}H activity (10^3 dpm)	Recovery (%)
Experiment A		
[^{3}H]Histone* plus postmitochondrial supernatant†	1.626	100
First DNA histone pellet	1.170	72
Second DNA histone pellet	0.357	22
140 m*M* NaCl washing of histone nucleates	0.018	1.2
Experiment B		
[^{3}H]Histone* added to cell lysate‡	1.610	100
Extraction of exogenous histones with low-molecular-weight DNA§		
First extract	1.251	77.7
Second extract	0.130	8.1
Third extract	0.113	7.0
Combined extracts clarified by centrifugation‖		
[^{3}H]Histones in supernatant	1.352	84.3
[^{3}H]Histones in pellet	0.136	8.5

*[^{3}H]Histones were prepared by reductive methylation (*48*). Twenty milligrams of duck erythrocyte total histone in 2 ml of 0.2*M* sodium borate, *p*H 9.0, was combined with 100 μl of 0.2*M* sodium borate containing 2.58 mg of NaH_4B and reacted with 3 mc of [^{3}H]formaldehyde (specific activity, 1 mc/10 μmole) in five sequential additions. The histones were then purified by gel filtration on Sephadex G-25 in 0.9*M* acetic acid. The specific activity of the purified histone was 6.5×10^6 count/min per milligram. †A suspension of lysed duck erythrocytes (5 ml) in a solution consisting of 0.1*M* KCl, 3.8 m*M* $MgCl_2$, 10 m*M* tris-HCl, *p*H 7.4, was centrifuged at 16,000*g* for 10 minutes. To the supernatant was added 100 μg of ^{3}H-labeled histone. Highly polymerized calf thymus DNA (Worthington) (200 μg dissolved in a solution consisting of 140 m*M* NaCl, and 10 m*M* sodium citrate) was then added, and the mixture was shaken for 30 minutes before centrifugation in a SW 65 Ti rotor at 60,000 rev/min for 30 minutes. The pellet was saved, and the supernatant was treated with an additional 100 μg of DNA. After centrifugation, the two pellets were combined and washed extensively with 140 m*M* NaCl. Samples of all fractions were digested with Protosol and radioactivity was counted in a toluene-Liquifluor mixture. ‡One milliliter of frozen duck erythrocytes (about 7×10^9 cells) was allowed to thaw, and 100 μg of [^{3}H]histone was added. §Low-molecular-weight DNA was prepared by sonication in a Branson sonifier for five 1-minute intervals. The DNA (1.4 mg dissolved in 5 ml of a solution of 0.25*M* sucrose, 40 m*M* NaF, and 10 m*M* tris-HCl, *p*H 7.4), was added to an equal volume of cell lysate in a solution consisting of 0.25*M* sucrose, 1 m*M* $MgCl_2$, 50 m*M* $NaHSO_3$, 40 m*M* NaF, and 50 m*M* tris-HCl; the mixture was homogenized and centrifuged at 1,100*g* for 10 minutes. The supernatant was saved, and the pellet was treated twice more by the same procedure. The three supernatants were clarified by centrifugation, and the histones were recovered as described for experiment A. ‖Histone nucleates were sedimented at 17,000*g*.

duction in the net positive charge of the molecule. Only some of the H4 molecules are modified in this way. The precise sites of acetylation of duck erythroblast H4 have not been determined but, on the basis of previous analyses of histone H4 from sources as diverse as calf thymus (*28*), pea seedlings (*28*), and trout testis (*30*), the major sites are likely to be lysine residues at positions 16, 12, 8, and 5 of the polypeptide chain.

The postsynthetic processing of histone H4 in the cell nucleus is further complicated by the fact that the phosphorylated form II is also subject to multiple acetylation. This gives rise to a subset of histone H4 derivatives of characteristically retarded electrophoretic mobility which contain zero, one, two, or three, or more acetyllysine residues in addition to the *O*-phosphorylated, *N*-acetylated serine residue at the amino-terminal end of the molecule.

All of these phosphorylated and acetylated derivatives of histone H4 can be detected after labeling erythroblast cultures with the appropriate radioactive precursors. Figure 10 shows the characteristic separations obtained when histone H4 molecules labeled with [^{32}P]orthophosphate and [^{3}H]acetate are subjected to high-resolution electrophoresis (*43*). The peak positions of the acetylated but not phosphorylated forms of the molecule do not coincide with the peak positions of the H4 molecules that are both acetylated and phosphorylated (Fig. 10b). At least nine forms of histone H4 exist in the erythroblast nucleus.

The complexity of histone H4 metabolism is further increased by the methylation of lysine residue 20, an event that follows histone synthesis (*51*) and which may strengthen histone interactions with DNA (*52*).

The acetyl groups on histone H4 lysine residues are subject to a rapid "turnover" (*11, 12*), and the balance of acetylation and deacetylation reactions determines the proportions of the H4 subfractions actually present in a given cell type. The kinetics of interconversion of individual histone subfractions have not been studied in the necessary detail in any cell type, but there are indications, both in the erythroblast and in other systems (*11, 13, 14*), that not all H4 molecules participate equally in acetyl group exchange.

Summary and Conclusions

The model shown in Fig. 11 summarizes our current view of the modifications of histone H4 in the cytoplasm and its subsequent processing in the cell nucleus. The cytoplasmic modifications include two types of acetylation, one producing ϵ-*N*-acetyllysine and the other producing *N*-acetylserine (*24, 25*). The phosphorylation of cytoplasmic H4 molecules involves the amino-terminal serine residue.

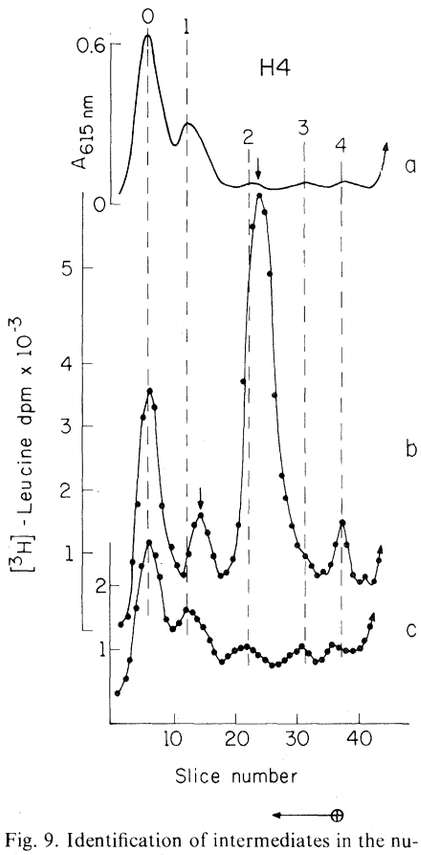


Fig. 9. Identification of intermediates in the nuclear processing of histone H4 molecules synthesized and modified in the cytoplasm. Bone marrow cells from anemic ducks were labeled with [^{3}H]leucine for 1 minute and incubated in unlabeled medium for 1 minute or for 1 hour. The cells were washed with a solution of 130 m*M* NaCl, 5.2 m*M* KCl, and 7.5 m*M* $MgCl_2$ and flash-frozen in Freon-12 chilled to its freezing point with liquid nitrogen. The cells were lyophilized and the nuclei were isolated under nonaqueous conditions (*50*) to preclude any exchange of radioactive histone molecules between the cytoplasm and the nucleus during isolation. The dried cells (100 mg) were suspended in 4.5 ml of cold glycerol and homogenized in a Sorvall Omnimixer for 4 to 5 minutes at 20,000 rev/min. The homogenate was overlaid on 1 ml of 15 percent 3-chloro-1,2-propanediol in glycerol and centrifuged in a SW 65 Ti rotor at 31,500 rev/min for 30 minutes at 4°C. The nuclear pellet was washed with a solution of 50 m*M* NaCl, 50 m*M* $NaHSO_3$, 40 m*M* NaF, and 40 m*M* tris-HCl, *p*H 7.0, and the histones were extracted in 0.25*N* HCl, purified on Bio-Rex 70, and analyzed by electrophoresis in 11 percent polyacrylamide gels. (a) Densitometric tracing of the bands in the H4 region of the gel. (b) Distribution of radioactive H4 molecules recovered from the nucleus after incubation for 1 minute with [^{3}H]leucine. Newly synthesized modified forms have lower mobility than the unmodified H4. (c) Distribution of radioactive nuclear histones after incubation for 1 minute in labeled medium followed by incubation for 1 hour with unlabeled medium. Nuclear processing of the recently synthesized H4 molecules has eliminated the characteristic phosphorylated and acetylated forms of cytoplasmic H4 and has generated the unmodified and multiply acetylated H4 subfractions in the proportions typical of the erythroid cell population.

After their entrance into the nucleus, the newly synthesized H4 molecules are subject to "early processing" by enzymes that remove the acetyl group from the modified lysine residue and hydrolyze the phosphate from the amino-terminal serine hydroxyl group. At the present time it is not known whether all of the newly synthesized H4 molecules undergo both elimination reactions. The acetyl group on the serine remains.

Later modifications of the nuclear H4 histone include the stepwise acetylation of lysine residues at specific sites in the polypeptide chain. Similar modifications of the phosphorylated H4 derivative may also occur. The model does not specify which of the lysine residues are acetylated at each step in the nuclear processing of histone H4. Although the lysine-16 residue is the major site of H4 acetylation, it is not known whether acetate uptake at this site precedes or follows acetylation at other lysine residues. (The model does not depict later metabolic changes involving the methylation and dimethylation of the lysine -20 residue.)

This complex series of events in the biosynthesis of histone H4 and its subsequent organization into the chromosome is in accord with a number of earlier observations. For example, the presence of histone–coenzyme A acetyltransferases have been reported in the cytoplasmic fraction of rat liver (*53*) and calf thymus (*54*). The cytoplasmic enzyme of calf thymus acetylates only histone H4, whereas the nuclear enzyme acetylates both H3 and H4 (*54*). Protein kinases that are ATP-dependent have been detected in the cytoplasm of many cell types (*55*). Thus, enzymes capable of acetylating and phosphorylating nascent histone chains are present in the cytoplasm.

The phosphorylation of histone H4 in avian erythroblasts has been reported (*56*). Our studies indicate that this is primarily a cytoplasmic event and that the phosphate groups are rapidly removed after the histone enters the nucleus. Virtually complete removal of previously incorporated [^{32}P]phosphate occurs within 20 minutes. Therefore, it is not surprising that phosphorylated forms of histone H4 do not accumulate in erythroblast nuclei in quantities sufficient for their detection by ordinary chemical or electrophoretic methods. The more sensitive isotopic labeling tech-

niques easily reveal a variety of phosphorylated and acetylated forms of the molecule (Fig. 10).

Fundamental differences exist between these two types of histone modification, depending on the intracellular localization of histone H4 and on the stage of erythrocyte differentiation. In the erythroblast, both acetylation and phosphorylation occur during H4 synthesis in the cytoplasm, and these cytoplasmic reactions appear to be tightly coupled; but the acetylation of histone H4 in the nucleus is not coupled to phosphorylation.

In the differentiation of avian erythroid cells, the phosphorylation of histone H4 is apparently limited to the dividing erythroblast stage, and it ceases when histone synthesis stops (*39*). The acetylation of histones H4 and H3 continues long after this stage, but it declines gradually as the red cells mature and lose their capacity for RNA synthesis (*20, 39, 57*).

The close correlations between histone H4 synthesis and its phosphorylation reported here are in good agreement with the findings of Dixon and co-workers (*58*) that the phosphorylation of histones in trout testis occurs in primary spermatocytes engaged in rapid DNA and histone synthesis rather than in mature cell types that have ceased the synthesis of histones.

Isotopic labeling techniques reveal multiple forms of histone H4 that are both acetylated and phosphorylated (Fig. 10). All these forms are present in very small amounts in isolated nuclei, and it is not clear whether phosphorylation of H4 can take place in the nucleus as well as in the cytoplasm of the dividing erythroblast. De-

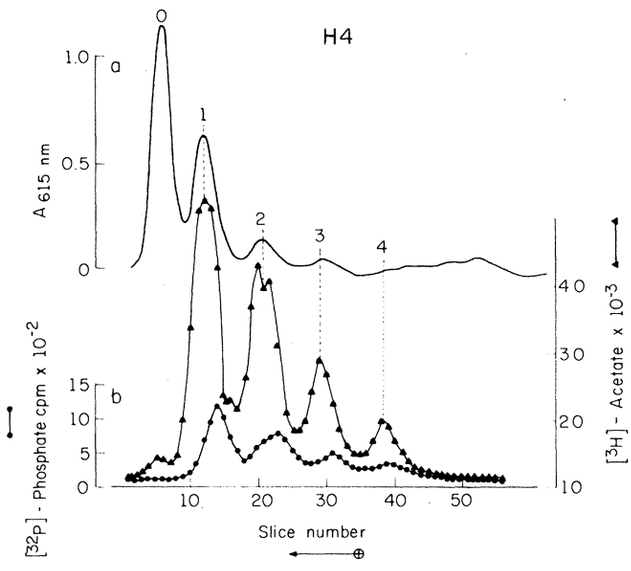


Fig. 10. Comparison of the electrophoretic mobilities of the phosphorylated and acetylated forms of histone H4. Bone marrow cells from anemic ducks were incubated in the presence of [^{32}P]orthophosphate (1.5 mc/ml) for 60 minutes and then [^{3}H]acetate (200 μc/ml; specific activity, 1 c/mmole) for 20 minutes. The histones were extracted in acid, purified on Bio-Rex 70, and separated by Bio-Gel P-60 chromatography. The H4 peak was collected and analyzed electrophoretically on 11 percent polyacrylamide gels (0.6 by 45 cm). (a) Densitometric tracing of the H4 bands; (b) distribution of the isotopically labeled histone molecules. The peak positions of the phosphorylated forms (•) do not coincide with the positions of the acetylated H4 subfractions (▲).

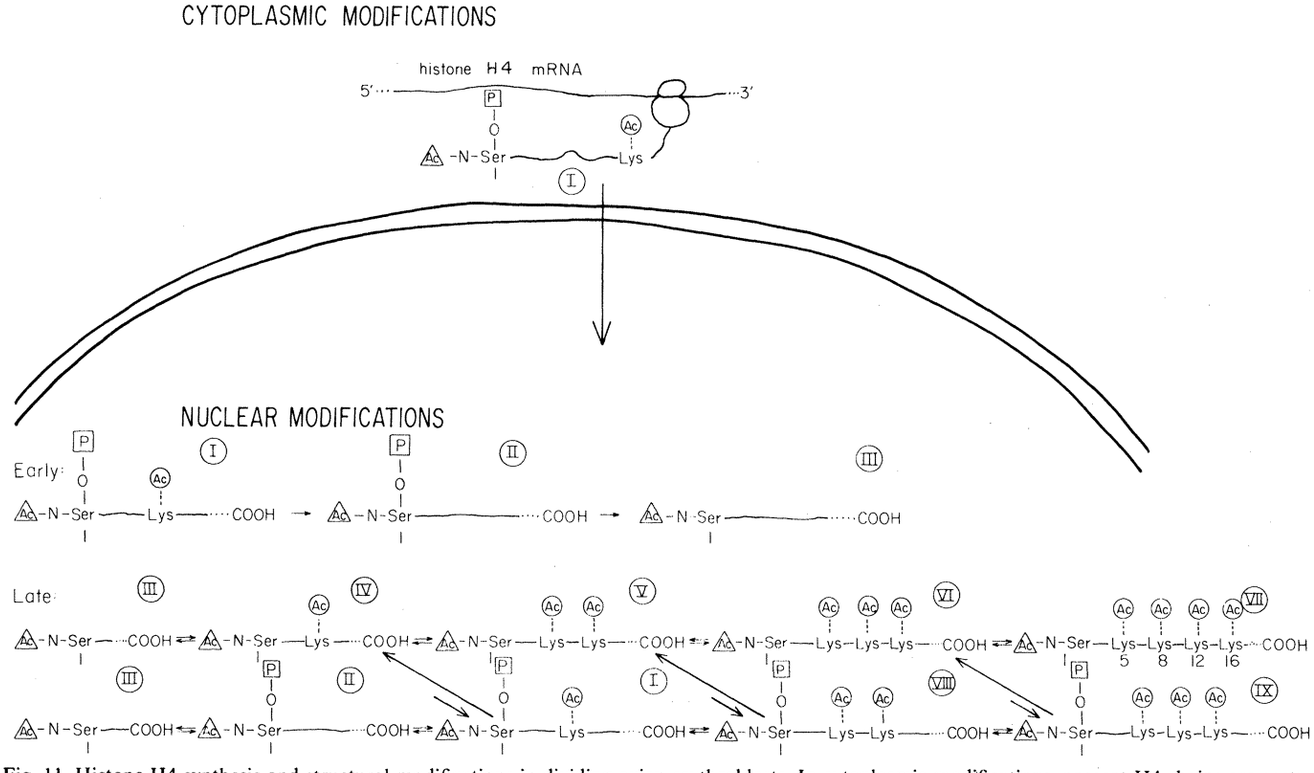


Fig. 11. Histone H4 synthesis and structural modifications in dividing avian erythroblasts. In cytoplasmic modifications, nascent H4 chains are acetylated at the amino-terminal serine residue and at a lysine residue within the polypeptide chain. The terminal serine residue is also phosphorylated. In nuclear modifications, newly synthesized and modified H4 molecules (form I) entering the nucleus from the cytoplasm are deacetylated (form II) and dephosphorylated (form III). Later modifications of form III include the sequential acetylation of lysine residues at four positions in the polypeptide chain, giving rise to forms IV, V, VI, and VII. The phosphorylated form II can also be acetylated to produce forms I, VIII, and IX. All of these nine forms have been detected in the nuclei of avian erythroid cells. The acetylation reactions involving lysine residues are reversible and allow the interconversions of the various modified forms as indicated by the arrows.

spite the rapid rate of dephosphorylation of newly synthesized H4 molecules (shown by the kinetic studies in Fig. 3), it is quite possible that some of the "new" H4 molecules (which were phosphorylated at the time of their synthesis in the cytoplasm) become acetylated in the nucleus before they lose their phosphate groups. Alternatively, the phosphorylation of the amino-terminal serine residue of histone H4 could reoccur in the nucleus and involve the various acetylated and nonacetylated forms of the molecule.

Recent evidence indicates that histone H4 may contain phosphoamide as well as phosphoester linkages (*59*). If such modifications take place in the erythroblast, they would not have been detected in the experiments described here, because of the lability of phosphoamide bonds under the acid conditions employed for the extraction and electrophoretic analysis of the histones.

Only a small fraction of the newly synthesized H4 molecules in the avian erythroblast ever occurs as the tetraacetylated form (even after incubation in unlabeled medium lasting up to 12 hours), because as proposed, multiple acetylation of the molecule would allow the DNA-binding regions to assume an α-helical configuration and permit a more controlled and precise "fit" of the basic protein into the wide groove of the DNA helix (*30, 32*). However, there is no indication that *all* newly synthesized H4 molecules of the erythroblast go through a tetraacetylated state. In fact, at no time after short-period labeling with [^{3}H]leucine do a majority of the newly synthesized H4 molecules in the nucleus occur as the multiacetylated derivatives.

An important distinction exists between histone H4 and other major histone classes synthesized at the same time. The cytoplasmic modifications of histone H4 include acetylation and phosphorylation, both of which affect the charge and electrophoretic mobility of the newly synthesized molecules. No corresponding alterations in mobility have been observed for the nascent H3 chains, nor is there any indication of ^{32}P labeling of H3 molecules recovered from the cytoplasm. These distinctions serve to emphasize the range, diversity, and complexity of structural modifications of the nuclear basic proteins at times of DNA replication and chromatin assembly.

It has been proposed that alterations of histone charge and structure brought about by acetylation and phosphorylation are part of the mechanism for modulating the interactions between histones and DNA (*11, 12, 30, 32, 33, 60*). The experiments described on the unique and transient modifications of histone H4 in dividing erythroblasts can be placed in that context. (In considering other possible functions for the rapid modifications of histone H4, we do not think it likely that these phosphorylation and acetylation reactions play a role in the transport of newly synthesized histone molecules from the cytoplasm to the nucleus, because the modified forms of histone H4 are found in isolated nuclei and because histone H3 does not undergo similar modifications.) We propose that the early modifications of histone H4 may be involved in a "nucleation" step during the assembly of chromatin particles on newly replicated DNA. The transient forms of H4 may bind DNA in a manner that favors the cooperative interaction with other histones to form the multimeric histone complex and its enveloping DNA strand. This model for the stepwise assembly of the chromatin subunit would be analogous to models proposed for the assembly of the ribosomal subunits; for example, *Escherichia coli* ribosomal proteins S4 and S20 interact with 16*S* RNA in a manner that favors subsequent binding of other proteins of the 30*S* ribosomal subunit (*61*). Although the best characterized modifications of histone H4 occur in regions of the molecule that interact with DNA, it is also possible that these changes affect the conformation and binding affinities of other regions of the molecule that are involved in histone-histone interactions. (It remains an open question whether other, nonhistone proteins, capable of recognizing the modified forms of the histones, also play a role in the correct assembly and spacing of the histone complexes on the growing DNA strands.)

The phosphorylation of histone H4 is linked to its synthesis in the cytoplasm, but the acetylation of H4 proceeds in the nucleus long after its synthesis has ceased. What is the significance of the continuing uptake and release of acetyl groups on H4 and other DNA-associated basic proteins? A large number of temporal and spatial correlations between histone acetylation and RNA-synthetic activity have been noted, but there is no simple quantitative relation between the proportions of acetylated histones and the number of active genes. For example, in calf thymus lymphocytes approximately half of the H4 molecules are acetylated, but transcription is limited to only a few percent of the genome. The multiply acetylated forms of H4 are present in small amounts, but it is not yet known whether these forms are localized at or near the template-active regions of the DNA.

If histones interact with DNA in sets, as appears to be the case, the modification of any one histone molecule may not be sufficient to alter the conformational state or DNA binding of the entire chromatin subunit—but multiple acetylations involving several histones in the complex would be expected to modify the electrostatic bonding of the multimeric histone aggregate to the enveloping DNA strand. The weakening of the associations between the basic regions of the histones and the polynucleotide chain could lead to reversible dissociation of the DNA-histone complex, freeing the DNA for transcription.

In this model, chromatin subunits can differ in the nature and extent of acetylation in their component histones. [An analogous model is the assembly of subunits of the bacterial enzyme, glutamine synthetase, in which individual subunits may or may not be adenylated, and the activity of the enzyme complex depends on the overall level of adenylation (*62*).] Increased acetylation of the histones is presumed to permit "slippage" or release of the DNA strand. This would account for the general correlation between acetylation of the histones and a diffuse, active state of the chromatin.

Finally, the enzymatic modification of histones is one of the most dynamic as-

Table 3. Amino acid composition of a [^{32}P]phosphate- and [^{3}H]lysine-labeled histone H4 peptide from duck erythroid cells. Histone H4 was prepared from the nuclei of cells labeled with [^{32}P]orthophosphate for 1 hour and with [^{3}H]lysine for 1 minute. The H4 was obtained in pure form by Bio-Gel P-60 chromatography. The histone was digested with chymotrypsin (*28*). Two peaks containing both ^{32}P and ^{3}H activity were recovered by chromatography on Sephadex G-25, and the peptides were further separated by electrophoresis in 18 percent polyacrylamide gels. The resulting bands were analyzed for radioactivity. A fast-moving band containing both isotopes and having a mobility much greater than that of the peptide containing residues 1 to 37 (*64*) was selected for analysis.

Amino acid	Composition of peptide (residues 1 to 22)	
	Observed* (nmole)	Calculated† (number of residues)
Serine	1.7	1
Glycine	8.0	8
Arginine‡		3
Lysine	3.9	4
Methyllysine§		1
Leucine	2.2	2
Histidine	0.7	1
Valine	0.8	1
Alanine	1.5	1

*The amino acid composition of the leading peptide band (recovered from parallel gels) was determined by acid hydrolysis and microanalysis on a Durrum amino acid analyzer. The serine figures were corrected for hydrolytic losses. †Number of residues expected from the known amino acid sequence of the corresponding peptide from calf thymus histone H4 (*28*) (Fig. 1). ‡Arginine was difficult to quantitate because of a large ammonia peak. §Methyllysine was not determined separately. Traces of aspartic and glutamic acids, probably representing minor contaminant peptides, were also detected.

pects of nuclear activity in dividing and nondividing cells. These reactions modify the structure and charge of proteins that are known to interact with DNA. In describing new complexities in the modifications of a particular histone, H4, we reemphasize the point that such post-synthetic modifications of protein structure usually signify the existence of control mechanisms for the regulation of subunit interactions and activity of organized enzyme systems.

Appendix

The presence of phosphorylated forms of histone H4 in avian erythroblasts has been confirmed by electrophoretic and chemical techniques. For example, it has been shown by Sung and Dixon (*30*) that the phosphorylated forms of trout testis H4 migrate much more slowly in starch-urea-aluminum lactate gels (*63*) than do the nonphosphorylated forms, regardless of the degree of acetylation of the latter. Our analysis by this procedure of histone H4 extracted from duck erythroblasts that had been doubly labeled with [^{32}P]orthophosphate and [^{3}H]lysine showed the presence of ^{32}P in slow-moving bands trailing the acetylated H4 subfractions. Since the same slow-moving ^{32}P-containing bands were labeled with [^{3}H]lysine after a 1-minute exposure, we conclude that the newly synthesized H4 molecules are phosphorylated.

Analysis of ^{32}P-labeled H4 (after its chromatographic purification on Bio-Gel P-60) showed that 98.4 percent of the ^{32}P activity was released as alkali-labile phosphate by treatment with 1*N* NaOH at 100°C for 15 minutes. This release suggests the presence of a phosphoester bond in the newly synthesized histone.

More definitive evidence on the nature of the bond was obtained by peptide analysis of the ^{32}P-labeled protein. Treatment of histone H4 with chymotrypsin cleaves the molecule into fragments of known amino acid sequence (*28*), which can be separated by ion-exchange chromatography (*64*). Chymotryptic cleavage of ^{32}P-labeled H4 from duck erythroblasts releases peptides bearing the isotopic phosphate. The smaller of these ^{32}P-labeled peptides was purified and subjected to amino acid analysis. The results (Table 3) confirm its localization at the amino-terminal region of the polypeptide chain (Fig. 1). Since this sequence (residues 1 to 22) contains only one esterifiable hydroxyl group—on the serine-1 residue—we conclude that the amino-terminal serine residue of H4 is subject to phosphorylation in erythroid cells engaged in histone synthesis. Evidence that this modification takes place in the cytoplasm was presented above (see Figs. 6 and 7). This conclusion is based on the assumption that the amino acid sequence of this region of histone H4 is the same in duck erythroblasts as it is in calf thymus (*28*), a reasonable assumption in view of the great evolutionary stability of this class of histones (*1a*). The fact that the NH_2-terminal serine residue of histone H4 can be phosphorylated was shown by Dixon and co-workers (*30*) in an analysis of histone structural modifications during spermiogenesis in the trout.

Identification of ϵ-*N*-acetyllysine in newly synthesized H4 molecules is complicated by the fact that "old" histone molecules are also subject to acetylation of their lysine residues. As a result, electrophoretic analysis of the nuclear histones after double labeling with [^{3}H]acetate and [^{32}P]orthophosphate shows overlapping of the [^{3}H]acetate in peaks corresponding to "old" and "new" (phosphorylated) H4 molecules (Fig. 10). To circumvent this difficulty, we have labeled only the newly synthesized histones with [^{3}H]lysine and then analyzed for the presence of ϵ-*N*-acetyl[^{3}H]lysine in the protein digest. The histones were labeled with [^{3}H]lysine in the cell-free protein-synthesizing system described in the legend to Fig. 6. After 10 minutes, the newly synthesized histones were precipitated by the addition of highly polymerized DNA and purified as described in the legend to Fig. 6. The ^{3}H-labeled histones were digested with trypsin and Pronase to release ϵ-*N*-acetyllysine (*27*). The latter was separated from free [^{3}H]lysine by ion-exchange chromatography (*27*). The modified amino acid was identified by cochromatography with an authentic ϵ-*N*-acetyllysine standard and by the presence of the [^{3}H]lysine label. Of the total histones synthesized in the erythroblast cytoplasmic system, only histone H4 has been found to be internally acetylated. Which of the lysine residues in histone H4 is modified remains to be determined.

References and Notes

1. In the new international nomenclature for histones adopted at the Ciba Foundation Symposium in London, April 1974 [E. M. Bradbury, *CIBA Found. Symp.* **28**, 1 (1975)], H4 replaces the older designations F2al, IV, GRK, and GAR; H3 replaces F3, III, and ARE; H2A replaces F2a2, IIb$_1$, and ALK; H2B replaces F2b, IIb$_2$, and KSA; Hl replaces F1, I, and KAP.

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above solution and stored at −80°C prior to isolation of the nuclei.
41. Nuclei were prepared at 4°C from frozen-thawed cells which were resuspended in 20 volumes of 10 m*M* tris-HCl buffer, *p*H 7.4, containing 40 m*M* NaF and 1 mM $MgCl_2$. After 3 minutes of hypotonic lysis, 2*M* NaCl was added to a final concentration of 100 m*M* and the cell lysate was centrifuged at 1100*g* for 5 minutes. The nuclear pellets were suspended in 20 volumes of a solution of 50 m*M* NaCl, 50 m*M* $NaHSO_3$, 40 m*M* NaF, 1 m*M* $MgCl_2$, 0.5 percent (by volume) Triton X-100, and 40 m*M* tris-HCl, *p*H 8.3; a Teflon-glass, motor-driven homogenizer was used. The suspension was centrifuged and the nuclear pellet was washed three times with 20 volumes of a solution of 50 m*M* NaCl, 50 m*M* $NaHSO_3$, 40 m*M* NaF, and 40 m*M* tris-HCl, *p*H 7.0, shearing in a Sorvall Omnimixer for 1-minute each time. The final pellet was extracted with 20 volumes of 140 m*M* NaCl prior to extraction of the histones. Sodium bisulfite was added as a protease inhibitor [S. Panyim, R. H. Jensen, R. Chalkley, *Biochim. Biophys. Acta* **160**, 252 (1968)].
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NEWS AND COMMENT

Solar Research Sweepstakes: States Vie for a Place in the Sun

From snowy New Hampshire in the north to American Samoa south of the equator, from Puerto Rico on the eastern approaches of the United States to Guam on the far western defense perimeter, from the urban centers of New York and California to the badlands of South Dakota, scientists and politicians are mobilizing to compete for the next big prize on the scientific horizon—the proposed Solar Energy Research Institute that is scheduled to get under way next year.

At this point it is a race without rules, for the federal Energy Research and Development Administration (ERDA), which will administer the institute, has not even decided what it will do, how big it will be (one guess is $50 million a year in operating expenses), what sort of site it will require, or even whether it will be one installation or scattered installations. But state and local officials are already pounding on ERDA's doors. Plagued with unemployment and bedeviled by rising fuel prices, they apparently believe a research center that might lead to an exotic new energy source and thriving spin-off industries is just the right antidote for their sagging economic fortunes. Not to mention the prestige and general intellectual uplift such centers are presumed to bring.

Even though the race has not yet officially begun, there are already dark hints that it might be rigged. These are based partly on the fact that President Ford, in off-the-cuff remarks at a 15 August energy symposium in Vail, Colorado, named only three states as possible sites, ignoring more than a score of other aspirants. Newspaper accounts said the President designated the three states as "front-runners," but the White House–edited transcript of the session indicates he was less emphatic. In answer to a question about the Administration's plans for solar energy, the President replied, according to the transcript:

> I was talking to Bob Seamans [the head of ERDA] a few days ago, and they have made significant progress. There is, unfortunately, competition developing between Arizona, New Mexico and Florida where the Federal Government will establish a solar energy research center. I do not know what the decision is going to be on what state gets that facility, but I am only using it as an example to point out that we mean business in this area.

Still, the fact that three states and only three states had seeped into the presidential consciousness caused cries of anguish from several competing states. Some of the most vociferous complaints came from Colorado, which was not only the site for the President's remarks, but also has launched one of the most vigorous efforts to land the new institute.

Administration aides later tried to soothe the ruffled competitors by explaining that President Ford had simply tossed out three names that occurred to him (or to a presidential assistant); they said there was no implication that any of the three was predestined to win the competition. Meanwhile, ERDA officials insist that they will be the ones who choose the final site and that they will do so on the merits of the case, unhampered by political interference.

The Solar Energy Research Institute—