Two Classes of Histone Acetylation in Developing Mouse Mammary Gland*

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SUMMARY
Mouse mammary gland explants rapidly incorporated 
\(^{14}\)C-acetate into the Fl, F2a1, F2a2, and F3 histones. These
acetylated histones were divided into two classes which were
distinguished by their metabolic stability, electrophoretic
mobility, and sensitivity to cycloheximide. The acetylation
of the F1 and F2a2 histones was inhibited by cycloheximide,
ocurred only during their synthesis, and was limited to the
\(\alpha\)-amino group of the terminal serine. In contrast, the pre-
formed F3 and F2a1 were acetylated on the \(\varepsilon\)-NH\(_2\) group of
internal lysine residues. This acetate was unstable, and
the histones were rapidly deacetylated. The F2b histone
did not incorporate \(^{14}\)C-acetate.

These results suggest that amino-terminal acetylation of
the F1, F2a1, and F2a2 histones represents a mechanism of
polypeptide chain initiation in eucaryotic cells analogous to
aminoterminal formylation in procaryotic cells.

If acetylation of histones has a functional role by modify-
ing the DNA-histone interaction, it is reasonable to assume
that this modification must also be reversible. Since the
acetylation of both F1 and F2a2 histones occurs only during
their synthesis, and this acetate remains with the stable his-
tone, it is unlikely that these proteins function in a reversible
DNA association. In contrast, both the F2a1 and F3 histone
fractions are reversibly acetylated on preformed molecules,
suggesting that acetylation of these proteins could function
in the control of RNA transcription.

The possibility of control of RNA transcription by specific
histone-DNA interactions is restricted, in that only a limited
number of histone classes (i.e. 15 to 20) have been observed (1–3).
In addition, at least one of these (F2a1) has a similar sequence
from widely different sources (4). For this reason, some investigat-
gors (5, 6) have recently turned their attention to specific
biochemical modifications which could result in altered histone-
DNA charge interactions as a reasonable mechanism to impart
at least some specificity in the control of RNA transcription. For
example, Vidali, Gershey, and Allfrey (7) have shown that
acetylation on the \(\varepsilon\)-NH\(_2\) group of lysine is an enzymatic process
that is mediated through acetyl coenzyme A occurring on pre-
formed histone molecules in the nucleus. If this process is
meaningful in transcription control, it must also invoke a rapid
deacetylation of these preformed histones. However, no
evidence of amino-terminal acetylation was observed in these
studies using isolated thymus nuclei, in spite of the fact that
the terminal amino acid of three of the histone classes (F1, F2a1,
F2a2) is acetylated (8, 9).

In the studies presented here, we have used the hormone-
mediated differentiation of mammary gland epithelial cells (10)
to further delineate the process of histone acetylation. This
in vitro system is particularly useful, in that it permits the care-
ful evaluation of the process of acetylation and deacetylation,
both of newly synthesized and preformed histones. Two sepa-
rate classes of acetylation have been observed and defined by
their behavior on acrylamide gel electrophoresis, their metabolic
stability, and their sensitivity to inhibitors of protein synthesis.

MATERIALS AND METHODS

Chemicals—\(^{1-14}\)C-Acetate (60 mCi per mmole) and \(^{3}\)H-lysine
(200 mCi per mmole) were purchased from New England Nu-
clear. Insulin was obtained from Squibb. \(N\)-Acetylserine and
O-acetylserine were obtained from Calbiochem. \(\varepsilon\)-N-Acetyl-
lysine was obtained from Sigma.

Organ Culture—Mammary glands were obtained from nullipar-
ous mid-pregnant C57Bl/6J mice (10 to 14 days) and cultured
on minimal Eagle's medium as previously described (11). Non-
essential amino acids (Microbiological Associates, Bethesda,
Maryland) were added to the medium at a concentration of
0.2 mM to reduce labeling of aspartic and glutamic acid by \(^{14}\)C-
acetate.

Labeling of Histones— Cultures were labeled for 60 or 90 min
with 10 \(\mu\)Ci per ml (0.10 mCi) of \(^{1-14}\)C-acetate (90 \(\mu\)Ci per
mmole). All explants were incubated on the medium described
above for 16 to 20 hours prior to hormone addition. Chase
experiments were performed by washing the tissues in media
and then transferring them to fresh media for 90 min. Where
noted, cycloheximide was present at 10 \(\mu\)g per ml, having been
added 10 min before the pulse was initiated.

Histones were labeled with 10 \(\mu\)Ci per ml (0.05 mCi) of \(^{3}\)H-
lysine (200 mCi per mmole) by incubation on Eagle's medium
The arginine-rich histones in the supernatant (F3, F2a2, F2al) and F2b, were then re-extracted from the residue with 1 ml of 0.15 M NaCl for 10 min at 3,000 x g. The clear pellet was recovered and suspended in 3 ml of 0.25 M sucrose, pH 7, and centrifuged for 2 hours at 20,000 rpm in the Spinco SW 25.3 rotor. The homogenate was layered onto a discontinuous gradient composed of 8 ml of I.95 M sucrose, 1.5 mM calcium acetate, and 9 ml of 1.6 M sucrose containing 1.5 mM calcium acetate (12). The homogenate, 0.5 ml, was layered onto each gradient and centrifuged for 2 hours at 20,000 rpm in the Spinco SW 25.3 rotor. The clear pellet was recovered and suspended in 3 ml of 0.15 M NaCl, 0.015 M Na\(^2\) citrate, pH 7, and centrifuged for 10 min at 3,000 x g. The pellet was then extracted with 1 ml of 0.25 N HCl for 18 hours. The histones in the supernatant were precipitated with trichloroacetic acid (final concentration 20%), and the precipitate was collected by centrifugation, washed in acid acetone (0.1 ml of 12 N HCl in 100 ml of acetone), and dissolved in a small amount of water. Histones were recovered by precipitation with 20% trichloroacetic acid.

Histones were fractionated into lysine- and arginine-rich fractions by the method of Johns (13). Chromatin was extracted for 18 hours with 1 ml of 0.25 N HCl in 80% ethanol. The arginine-rich histones in the supernatant (F3, F2a2, F2a1) (Fraction I) were recovered by precipitation with 4 ml of acetone at -20° overnight. The lysine-rich histones (Fraction II) F1 and F2b, were then re-extracted from the residue with 1 ml of 0.25 N HCl and recovered by precipitation with 20% trichloroacetic acid.

Fraction I was dissolved in 0.2 ml of 8 M deionized urea, containing 0.1 M phosphate buffer, pH 8.5, and 10\(^{-8}\) M dithiothreitol. After 30 min at 37°, 1 ml of 5 M urea, 0.1 M phosphate, pH 8.5, containing 5 mg of DTNB\(^1\) was added. After 1 hour, the solution was dialyzed exhaustively against distilled water. Histones were recovered by precipitation with 20% trichloroacetic acid. After this treatment, the F3 migrates as a single band on gel electrophoresis at pH 4.3, and is easily resolved from the F2a2 and F2a1 histones.

**Electrophoresis**—Electrophoresis was performed as described previously (11, 12) with the following modifications. Gels (10 x 0.5 cm) contained 20% acrylamide, 6.25 x urea, pH 4.3. A 1-em spacer gel was polymerized on top of the gel (12). Both the running gel and spacer gel were cross-linked with ethylene diamine (14) to allow the gels to be solubilized. Up to 50 μl of sample were applied, and electrophoresis was carried out for 12 hours at 1.5 ma per tube. Gels were stained for at least 5 hours in 1% Amido black and destained in 7% acetic acid. The gels were scanned with a Gilford gel scanner, frozen on Dry Ice, and sliced into 1-mm slices with a manual slicing device for counting (11). The slices were hydrolyzed in 0.2 N NaOH, neutralized, and counted in Triton-toluene using liquid scintillation (15). After swelling, the gels were about 14 cm long; migration distances refer to the swollen gels. Staining was quantitated by integration of the gel tracing as described previously (11, 12). Purified rat liver histones, prepared by the methods of Johns (13, 16) from purified nuclei, were used as standards. Calf thymus F3 which had been reacted with DTNB and had given a single band on electrophoresis at pH 4.3 was used as a standard for this fraction. Protein was determined by the ninhydrin reaction (17) after basic hydrolysis and by quantitative amino acid analysis.

Histones were also analyzed by the SDS gel system of Shapiro and Maizel (18). Gels (10 x 0.5 cm) were 20% acrylamide cross-linked with 0.1% ethylene diamine. They were stained with 0.25% Coomassie blue and destained as described by Weber and Osborn (19). They were scanned at 542 μm with the gel scanner, sliced, and counted as above.

**Analysis of Labeled Amino Acids**—The histones from 100 to 150 mg of tissue were dissolved in 0.2 ml of 0.1 N (NH₄)₂CO₃. Trypsin, 0.5 μg, was added, and the solution was incubated at 37° for 18 hours; Pronase, 20 μg, was added, and incubation was continued for 24 more hours at 37° (7). The solution was lyophilized, and the residue dissolved in water and lyophilized again. The residue was dissolved in 0.5 ml of 0.01 N HCl. A solution of amino acids (0.5 μmole each of amino acids, 1 μmole of cysteic acid, 2 μmoles of N-acetylserine, 2 μmoles of e-N-acetylseryl-5, 0.5 μmole of norleucine), containing 10,000 cpm of ¹⁴C norleucine and ¹³C-arginine as internal standards, was added, and the sample was applied to a single long column of the Technicon amino acid analyzer. The effluent was passed through a Packard flow counter, and the stream was split, one-half to a fraction collector and one-half to analysis. N-Acetylseryl-5 was located by the ninhydrin reaction (17) after hydrolysis with 13% NaOH. Aliquots of the fractions were also counted in a Packard scintillation counter to precisely locate the peak.

**RESULTS**

Histones were resolved into three bands on electrophoresis at pH 4.3 (Fig. 1). The slowest migrating band was the F1 histone; the major band contained the F3, F2b, and F2a2 in a ratio of 7:5:2. The abbreviations used are: DTNB, dithiobisnitrobenzoic acid; SDS, sodium dodecyl sulfate.
Histones were prepared 20 hours after insulin treatment from explants incubated with $^{14}$C-acetate for 90 min. In one experiment total histones were prepared. In a second experiment, histones were fractionated into two classes as described under "Materials and Methods." The arginine-rich histones (C) were reacted with DTNB to prevent aggregation of the F3. The separation of the F3 and F2a2 histones observed in C is partly due to order of electrophoretic mobility, and the fastest migrating protein was the F2a2 (11). In some preparations, a small band was detected that migrated slower than F1 (Fig. 1). This represented a multimer of the F3 histone which, depending on conditions, may aggregate by forming disulfide bonds (20). The minor band, F1c (Fig. 1) is a very lysine-rich histone also recently observed and characterized by Panyim and Chalkley (21). The F3 monomer, F2b, and F2a2 were not resolved in this preparation. It should be noted that the F2a1 from the mammary gland is visually observed as two closely spaced bands under these conditions. Due to a slight curvature of the two F2a1 histones, the optical density gel scan distinguishes these only as a shoulder in the optical density tracing (Fig. 1, arrow). Electrophoresis of calf thymus F2a1 also revealed two bands of equal intensity, probably corresponding to the two equally occurring forms of the molecule, one with ε-N-acetyllysine in position 16, the other unmodified in this amino acid residue (22). A similar gel pattern for the F2a1 has also been reported by Panyim and Chalkley using a different gel system (2).

Our previous studies (11) have demonstrated that treatment of mouse mammary gland explants with insulin results in a coordinate synthesis of histones and DNA initiated 12 to 16 hours after insulin addition, reaching a maximum at 20 to 24 hours. Histone and DNA synthesis are stimulated 2- to 3-fold 24 hours after insulin addition. However, it is important to note that both protein (11) and RNA synthesis (23, 24) were stimulated much earlier, i.e. within 4 hours after insulin treatment.

Twenty hours after addition of insulin, a time of maximum histone synthesis, F3, F2a2 and F2a1 histones were labeled when the explants were exposed to $^{14}$C-acetate for 90 min (Fig. 2). In view of the fact that the specific activity of the F2b was reduced by re-extraction of the nuclei with 0.25 N HCl in 80% ethanol before extraction with 0.25 N HCl, it is probable that even the low activity in this fraction was due to contamination with a small amount of histones F3 or F2a2. Thus, very little, if any, acetate was incorporated into this fraction. The arginine-rich histones, F3 and F2a1, had the highest specific activity.

It is important to note that the $^{14}$C-acetate incorporated into the F1 histone under went co-electrophoresis with this histone (Fig. 2, O.D. 450 versus $^{14}$C), although the acetate that was incorporated into the F3 and F2a1 did not under go co-electrophoresis with the latter fractions (Fig. 2). These observations may be explained by the fact that the F3 and F2a1 molecules labeled with $^{14}$C-acetate differ in charge from the bulk of the histones. They also imply that only a small percentage of these histone molecules are acetylated, differentiating them from the bulk of the unmodified histone. In contrast, these studies suggest all molecules of the F1 histone were acetylated, as none of the labeled molecules differed in charge from the unlabeled F1 histones.

To determine if acetylation was dependent on protein synthesis, experiments were performed in the presence of cycloheximide (10 µg per ml, sufficient to inhibit protein synthesis >98%). In the presence of the inhibitor, acetate was still incorporated into the F3, F2a2, and F2a1, although no label was incorporated into the F1 (Fig. 3B). Since acetylation of the F1 was completely inhibited by cycloheximide, while radioactivity in the other fractions was only slightly reduced, acetylation of the F1 was specifically dependent on protein synthesis. It should be noted once again that the $^{14}$C-acetate-labeled histones, which were acetylated in the presence of cycloheximide, again migrated slower on electrophoresis than the bulk of the histones.
Fig. 3. Total histones were prepared 20 hours after insulin addition from explants incubated in the presence of 14C-acetate for 90 min. A, 90-min pulse; B, 90-min pulse in the presence of cycloheximide; C, 90-min pulse + 90-min chase. Electrophoresis conditions were those of Fig. 1. ---, O.D.414; -- --, cpm counts per minute per 1-mm slice.

Table I

Turnover rates of acetate in histone fractions

Histones were prepared 20 hours after insulin addition from explants incubated in the presence of 14C-acetate for 90 min. Histones were fractionated as shown in Fig. 2, B and C, and analyzed by quantitative gel electrophoresis.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>90-min pulse period with 14C-acetate</th>
<th>90-min chase period</th>
<th>Histone fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse</td>
<td>Cycloheximide</td>
<td>Chase</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>None</td>
<td>2140</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>1140</td>
<td>2140</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>None</td>
<td>1810</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>None</td>
<td>135</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

Table II

Effect of cycloheximide on acetylation

Histones were prepared from explants incubated for 90 min in the presence of 14C-acetate (10 μCi per ml) 20 hours after addition of insulin. Histones were analyzed by quantitative gel electrophoresis. The stained gels were sliced into 1-mm fractions and the specific activity of each band was determined. Chase experiments were performed by washing the labeled explants in fresh media and incubating for 90 min. Where indicated, cycloheximide was present at a concentration of 10 μg per ml.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pulse</th>
<th>Chase</th>
<th>F1</th>
<th>F2b, F2a2</th>
<th>F2al</th>
</tr>
</thead>
<tbody>
<tr>
<td>No hormone</td>
<td>90 min</td>
<td>None</td>
<td>1000</td>
<td>880</td>
<td>1440</td>
</tr>
<tr>
<td>Insulin 2 hours</td>
<td>90 min</td>
<td>90 min</td>
<td>1140</td>
<td>440</td>
<td>670</td>
</tr>
<tr>
<td>Insulin 20 hours</td>
<td>90 min</td>
<td>90 min</td>
<td>1400</td>
<td>1480</td>
<td>1710</td>
</tr>
</tbody>
</table>

Table III

Effect of insulin on acetylation

Histones were prepared and analyzed as in Table I. The labeling period was started after the indicated time in the presence of hormone. In experiments performed 2 hours after insulin addition, explants from one animal were incubated on normal media for 16 hours, and then insulin was added to one-half of them.

To determine the rate of turnover of the acetate that was incorporated into the histones, pulse-chase experiments were performed. Although the same histone fractions were labeled (Table I), both the radioactivity pattern on the gel (Fig. 3C) and the relative specific activities were altered.

As a result of the chase, the 14C-acetylated histones now migrated much closer to the bulk of the histones (Fig. 3C). In particular, there was now a greater distance between the F1 and 14C-labeled F3 molecules and a decrease in the histone fraction which on electrophoresis migrates immediately prior to the F2a1 molecules. The amount of label which migrated near the stained bands remained approximately constant, and the specific activity of the F3 + F2a2 and F2a1 bands was reduced (Table II) and presented no evidence of a precursor-product relationship. Rather, the data suggest that during the chase, 14C-acetate was selectively removed from the molecules with a lower positive
Fig. 4. Total histones were prepared 2 hours after insulin addition from explants incubated in the presence of 14C-acetate for 60 min. Cycloheximide was present during the labeling period. The 14C-acetate-labeled histones were mixed with 3H-lysine-labeled histones ("Materials and Methods") and subjected to co-electrophoresis. A small amount of non-histone protein (X) is seen on the right; left, 20% acrylamide gel, 6.25% urea, pH 4.3.

The specific activity of the F1 remained constant during the chase, while that of the other fractions was reduced (Table II).

Thus, three properties separate acetylation of the F1 from that of the other fractions: (a) metabolic stability, (b) sensitivity to cycloheximide, and (c) electrophoretic mobility. The F1 is thought to contain only NH2-terminal acetate (5), and these data suggested that NH2-terminal acetylation was distinct from internal acetylation. The data in Table II support this hypothesis.

The F2a2 and F2a1 histones are known to contain aminoterminal N-acetylserine (8). As can be seen, cycloheximide reduced labeling of the F2a2 and F2a1. In addition, a component of acetylation of these histones normally stable during a chase was prevented by cycloheximide (Lines 2 and 4, Table I), and this has some of the same properties as the acetylation of the F1. The data in Table I shows that the acetyl groups of the F2a2 histone were stable during a chase consistent with the concept that F2a2 histones contains primarily N-acetylserylene.

Deacetylation of the F3 and F2a1 occurred in the presence of cycloheximide, and thus was independent of protein synthesis, as was the acetylation.

As a further test of the hypothesis that stable amino-terminal acetylation was a process connected with histone synthesis, explants were labeled with 14C-acetate during a period when little histone synthesis was occurring, i.e. 2 hours after insulin treatment or after no hormone treatment. Under these conditions the same fractions were labeled, although the specific activities differed (Table III). Again the label in the F2a2 + F3 fractions and in the F2a1 fraction migrated slower than the bulk of the histone, while that in the F1 underwent co-electrophoresis with its unlabeled fraction (not shown). Cycloheximide again specifically prevented acetylation of the F1 (Fig. 4). The specific activity of the F1 histone was lower, both in the absence of insulin and after its presence for 2 hours, than in the samples treated for 20 hours. This was consistent with the concept of F1 acetylation and synthesis being related. In contrast, both 2 and 20 hours after insulin administration, acetylation of the F3 + F2a2 histones and the F2a1 histone was higher than in the untreated cultures. Chase experiments showed that the turnover of acetate in the F3 + F2a2 was rapid, both in untreated cultures and after 2 hours of insulin treatment, while that in the F1 was stable in all cases. The apparent decrease in turnover rates 20 hours after insulin treatment was probably due to increased amino-terminal acetylation of the F2a2 and F2a1 during the period of histone synthesis, as was seen for the F1, and not to any change in the rate of deacetylation of internal lysine residues. These data were also consistent with the hypothesis that metabolically stable amino-terminal acetylation was related to protein synthesis, while internal acetylation is independent of protein synthesis and stimulated by the presence of insulin.

To further compare the turnover rates of the acetate in different fractions, histones were fractionated 20 hours after insulin treatment as in Fig. 1 after a 90-min pulse and a 90-min pulse + 90-min chase (Table I). The acetate incorporated into the F1 and F2a2 histones was stable, suggesting that the F2a2 contains acetate primarily on the amino-terminal serine. The F3 had the highest specific activity, and the acetate in this fraction was most labile. The acetyl groups in the F2a1 were more
Histone Acetylation

Fig. 5. Histones were prepared from explants incubated in the presence of 14C-acetate. A, 2 hours after insulin addition, histones were digested for 90 min to amino acids with trypsin and Pronase, and analyzed on a Technicon long column amino acid analyzer. The effluent was monitored on a Packard flow counter (60% efficiency for 14C), and split into two streams, one used for ninhydrin analysis, and the other sent to a fraction collector (4-min fractions) for basic hydrolysis and ninhydrin analysis to detect N-acetylsine. To each sample, a standard amino acid mixture + N-acetylsine and c-N-acetylysine was added for 90 min followed by a 90-min chase, 20 hours after insulin addition. ----, O.D. 0.1%; ---, 14C-acetate; --, 3H-acetate after basic hydrolysis; C.A., cysteic acid.

Fig. 6. An aliquot of Sample A from Fig. 5 was analyzed by paper electrophoresis in 2.5% formic acid-8.7% acetic acid, pH 1.9, to separate e-N-acetyllysine from glycine. Electrophoresis was for 40 min at 400 volts.

stable. The faster turnover rate of the acetate in the F3 compared to that in the F2a1 was probably not due solely to the stable amino-terminal acetylation in the F2a1, as it was also observed in the presence of cycloheximide (Table II, Lines 3 and 5) and when histone synthesis was minimal (Table III).

Double label experiments were used to establish that the 14C-acetate which failed to undergo co-electrophoresis with the histones was actually associated with histones. Histones labeled with 3H-lysine as described under "Materials and Methods" were mixed with 14C-acetate labeled samples, both on pH 4.3, 6 M urea gels and on gels containing 0.1% SDS, 0.1 M phosphate, pH 7.1. The urea gel separated histones on the basis of size and charge, while the SDS gel separated solely on the basis of size. The 14C-acetylated histones migrated slower than the 3H-labeled histones in the urea gel (Fig. 4, left). However, on SDS gels (Fig. 4b), where separation was not affected by charge, the 14C and 3H underwent co-electrophoresis. The bands on the SDS gels were identified using pure rat liver standards as the F1 (a slow-moving double band), the F3 + F2b intermediate, and F2a2 + F2a1 (the fastest moving band). The 14C-sample used was prepared in the presence of cycloheximide so that the F1 was not labeled. The ratio of counts in the F3 + F2a2 peak in the urea gel to the F2a1 peak was similar to the ratio of the F3 to the F2a2 + F2a1 in the SDS gels (1.1 and 0.9) consistent with the observations above which suggest that the F2a2 contained primarily amino-terminal acetate and hence, would not be acetylated in the absence of protein synthesis. Only a small amount of contaminating protein (×) was seen in the SDS gel (Fig. 4).

To establish which amino acids were acetylated, the proteins were digested to their amino acids with trypsin and Pronase, and analyzed on an amino acid analyzer and a Packard flow scintillation spectrophotometer. In addition, since acetylated amino acids would be expected to give a negative ninhydrin reaction, the stream from the column was split, half going to a fraction collector. The fractions were hydrolyzed with base before performing the ninhydrin reaction. Two different samples were analyzed (Fig. 5). At the period of maximal histone
synthesis, 20 hours after insulin administration, the histones were labeled with \(^{14}C\)-acetate for 90 min followed by a 90-min chase. These preparations gave two major radioactive peaks using the Packard scintillation spectrometer scan from the amino acid analyzer; one at a position identical with that of N-acetylserine, and the second at the position of \(\varepsilon\)-N-acetyllysylsine (Fig. 5B).

If \(^{1}\text{H}^{14}\text{C}\)-acetate is incorporated into histones as described above during a period of minimal synthesis, \(\varepsilon\) at a period 2 hours after insulin administration, only one major peak was observed coincident with \(\varepsilon\)-N-acetyllysylsine (Fig. 5A). Thus, very little radioactivity was observed in the amino-terminal serine in the absence of rapid histone synthesis.

There was no evidence of radioactivity in the region of O-acetylserine in any histone preparation. Since \(\varepsilon\)-N-acetyllysylsine was not resolved from glycine, it was necessary to use paper electrophoresis at pH 1.97 to firmly establish that labeled glycine was not present (Fig. 6). Once again, this demonstrates that the major incorporation was in the \(\varepsilon\)-N-acetyllysylsine.

These experiments establish two classes of histone acetylation. One occurs on all molecules of histone F2a1, F2a2, and F1, on the amino-terminal serine. It is metabolically stable, sensitive to inhibitors of protein synthesis, and prominent during histone synthesis. The second, on the F3 and F2a1 histone, occurs on only a few molecules of a given class. It is insensitive to inhibitors of protein synthesis, metabolically unstable, and not associated with histone synthesis. It occurs on internal \(\varepsilon\)-amino groups of lysine.

**DISCUSSION**

While it has been known for some time from chemical evidence that some histones have acetylated amino-terminals (8, 9), this is the first report which characterizes the incorporation of acetate into the amino-terminal of histone molecules. The amino-terminal of the F1, F2a2, and F2a1 histone is N-acetylicerine (8). The NH$_2$-terminal of the F2b histone is proline, and that of the F3 is alanine (13). However, neither of these amino acids is acetylated. The characteristics and mechanism of amino-terminal acetylation differ from those of the \(\varepsilon\)-NH$_2$ group of internal lysines. The data presented here strongly suggest that amino-terminal acetylation occurs during histone synthesis, which presumably takes place in the cytoplasm (25, 26). It is suggested that, paralleling the bacterial mechanism of protein synthesis (27), the acetylated amino acid is involved in initiation of the polypeptide chain through a specific tRNA.

Recently, Liew, Haslett, and Allfrey (28) have described N-acetylicerine tRNA involved in synthesis of the F2a1 histone. It is well established that *Escherichia coli* proteins are initiated with N-formylmethionine, a blocked amino-terminal. In mammalian systems the situation is less clear. Hemoglobin is initiated with unblocked valine (29), while ovalbumin is initiated with N-acetylserine.

The F3 histone in mammary tissue reaches the highest specific activity and is most rapidly deacetylated. This observation is in agreement with our earlier results for HeLa cells (31), but differs from that of Pogo et al. (5) in regenerating liver. The acetate incorporated into the fraction has a half-life of less than 90 min. In contrast, the F2a1 incorporates acetate more slowly, and its deacetylation is slower than F3. The distinctive electrophoretic migration suggests that, at any one time, only a small percentage of F3 and F2a1 molecules are internally acetylated. It is unlikely that \(\varepsilon\)-N-acetyllysylsine is present to any extent in any other histone fraction, and there is no evidence of active deacetylation on the F2a2 histone. This concept is in agreement with data obtained with isolated calf thymus nuclei (7).

It has been suggested on the basis of indirect evidence (22) that the F3 histone contains O-acetylseryn. However, in agreement with studies on isolated nuclei (7), our observations show no evidence of O-acetylseryn.

The F2a1 histone has a positive charge of +21 (22), and the F3 histone has a maximum positive charge of +40, although the actual charge is probably less than this. Thus, acetylation of a single lysine would result in a 3 to 5% change in positive charge which is easily detectable in our electrophoretic system. The F2a1 is always resolved into two closely spaced bands, and the peak of acetylation is always on the slower moving band. The slower moving band stains with less intensity and corresponds to roughly 20 to 30% of the total F2a1 histone. In addition, F2a1 prepared from calf thymus migrates as two bands of equal intensity, and it is known that about 50% of these molecules contain \(\varepsilon\)-N-acetyllysylsine at position 16 (22).

Studies reported here suggest that there is less \(\varepsilon\)-N-acetyllysylsine present in mouse mammary gland F2a1. In this regard, it is interesting to note that some species' specificity for acetylation of the F2a1 has been reported by DeLange et al. (4), in that both the quantity of \(\varepsilon\)-N-acetyllysylsine and its position within the molecule vary in F2a1 histone prepared from pea bud. In addition to lysine 16, the pea bud is also acetylated on lysine Residue 8 (4).

The data presented here show that insulin stimulates acetylation of the F3 histone of mammary epithelial cells within 2 hours, well before stimulation of histone synthesis and amino-terminal acetylation. Stimulation of RNA synthesis is also an early event following insulin treatment (23, 24). Although this data is consistent with that of Pogo et al. (5, 33), the hypothesis is far from proven.

Acetylation of \(\varepsilon\)-NH$_2$ lysine residues occurs on only a few histone molecules of a given class. If acetylation is to play a specific regulatory role, a mechanism must exist whereby the acetylase can recognize specific molecules to acetylate. It is probable that the acetylation is determined not only by the primary structure of the histone, but also by its interaction with chromosomal RNA's and acidic proteins.

**REFERENCES**


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2 R. D. Cole, personal communication.
Two Classes of Histone Acetylation in Developing Mouse Mammary Gland
William F. Marzluff, Jr. and Kenneth S. McCarty


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