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# Acetylated histone H4 is preferentially associated with template-active chromatin

(DNase II/$Mg^{2+}$ solubility/two-dimensional gels/gene expression)

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**ABSTRACT** **Chromatin from trout testis at an early stage of development was digested with DNase II (deoxyribonucleate 3′-oligonucleotidohydrolase; EC 3.1.4.6), and the solubilized products were fractionated into $Mg^{2+}$-soluble and -insoluble components. An examination of the histones from these fractions by one- and two-dimensional polyacrylamide gels showed that the highly acetylated species of histone H4 (di-, tri-, and tetra-acetylated) were associated mainly with the $Mg^{2+}$-soluble material. Digestion of this chromatin fraction with pancreatic ribonuclease converted more than half of it to an insoluble state, and the acetylated H4 remained associated with the precipitated fraction. No changes in the other histones were noted, but two other basic proteins were also found to be associated with the $Mg^{2+}$-soluble fraction. Since this fraction is enriched in transcribing gene sequences, it is concluded that the histone H4 of active genes is present in a highly acetylated state.**

The four nucleosomal histones, H2A, H2B, H3, and H4, are acetylated at the ε-amino groups of specific lysyl residues (1–4). Only partial acetylation is ever observed at a given site, and this leads to charge heterogeneity in these proteins (1, 4). The location of acetylated lysyl residues in the most basic regions of these histones, as well as the reversibility of the acetylation process *in vivo*, has led to the suggestion that this modification may be important in the modulation of histone–DNA interactions (4–6).

Histone acetylation has been correlated with increased transcriptional activity in various systems (2, 7, 8), although direct evidence for the involvement of acetylation in gene activity is lacking. Recently, Gottesfeld and his coworkers have shown that the DNase II (deoxyribonucleate 3′-oligonucleotidohydrolase; EC 3.1.4.6) fractionation procedure of Marushige and Bonner (9), coupled with $Mg^{2+}$ precipitation, enables them to isolate a chromatin fraction enriched in actively transcribing sequences (10, 11). Levy-Wilson *et al.*, applying this procedure to *Drosophila* cells, observed a 40% increase in the incorporation of labeled acetate into histones of the template-active fraction (12). We have examined the patterns of histone acetylation in this fraction and we find that it is greatly enriched in the multiply acetylated forms of histone H4, as well as in two unidentified basic proteins.

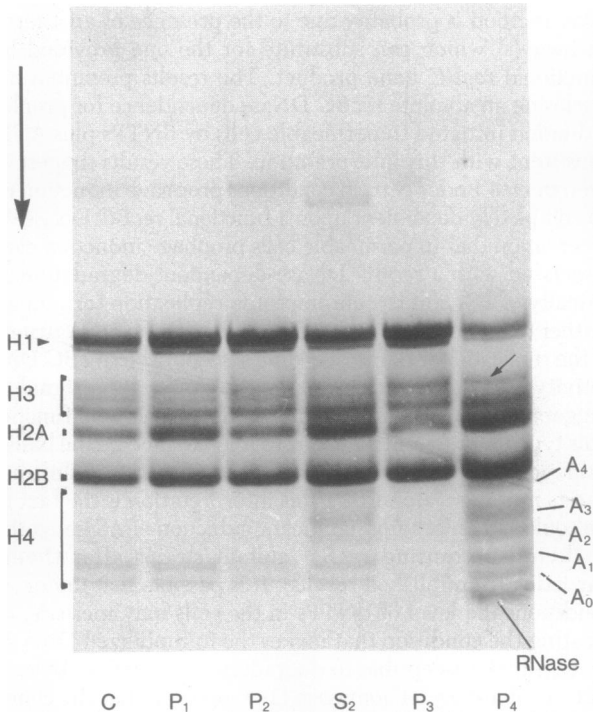


FIG. 1. Histone profiles on an acid/urea/polyacrylamide gel. Histones were prepared from chromatin fractions and separated on a 1.5-mm slab gel by the system of Panyim and Chalkley (14). The gel was stained with Coomassie blue. C, Histones from unfractionated chromatin control; $P_1$, histones from chromatin pellet remaining after DNase II digestion; $P_2$, histones from $Mg^{2+}$-insoluble fraction of DNase II-released material; $S_2$, histones from $Mg^{2+}$-soluble fraction of DNase II-released material; $P_3$, histones from fraction rendered $Mg^{2+}$ insoluble by the first RNase digestion of $S_2$; $P_4$, histones from fraction rendered $Mg^{2+}$ insoluble by the second RNase digestion of $S_2$. The positions of the histones are indicated in sample C. Histone H3 is resolvable into three components (unmodified, mono-, and diacetylated). H4 is resolvable into four components: $A_0$, unmodified; $A_1$, monoacetylated; $A_2$, diacetylated; $A_3$, triacetylated; $A_4$, tetraacetylated. RNase, pancreatic ribonuclease.

## MATERIALS AND METHODS

**Trout Testis.** Testes at early stages of development were obtained from naturally maturing rainbow trout (*Salmo gairdneri*) at the Sun Valley Trout Farm, Mission, British Columbia, and stored at −80° until use. Testis nuclei and chromatin were prepared essentially as described by Honda *et al.* (13), except that nuclei were treated with the protease inhibitor phenylmethylsulfonyl fluoride (0.1 mM) prior to chromatin preparation.

**Chromatin Fractionation and Histone Extraction.** Chromatin was digested with DNase II (Sigma) at 4 enzyme units per $A_{260}$ unit for 8 min at 24°, and fractionated according to Gottesfeld and Butler (11). The $Mg^{2+}$-soluble ($S_2$) fraction was divided into two portions, one of which was treated with 10 μg of pancreatic RNase per ml for 10 min at 37°, the other of which was treated for 20 min at 24°. After centrifugation the pellet ($P_3$) was saved, and the supernatant was redigested with

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Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

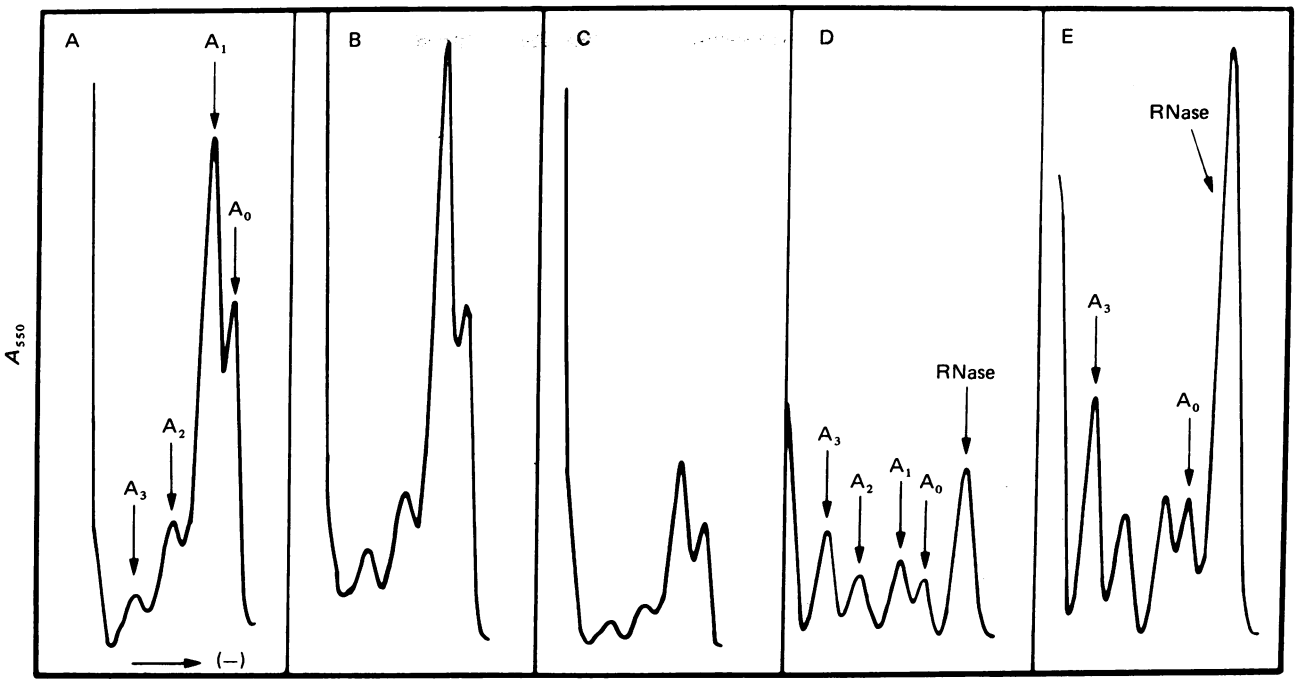


FIG. 2. Gel scans of the histone H4 regions of an acid/urea gel. The gel is from a different experiment from that illustrated in Fig. 1. $A_0$, $A_1$, $A_2$, and $A_3$ are subspecies of histone H4, as described in the legend to Fig. 1. (*A*) H4 from unfractionated control chromatin; (*B*) H4 from $P_1$, the chromatin pellet remaining after DNase II digestion; (*C*) H4 from $P_2$, the $Mg^{2+}$-insoluble fraction of DNase II released material; (*D* and *E*) H4 from $P_3$ and $P_4$, respectively, the fractions rendered $Mg^{2+}$ insoluble by RNase digestion of the $Mg^{2+}$-soluble fraction. Gels were stained with Coomassie blue and scanned in a Gilford spectrophotometer at 550 nm.

a further 10 $\mu$g of RNase per ml for 20 min (37°) or 40 min (24°). Before acid extraction of the histones, the $S_2$ fraction was concentrated with an Amicon ultrafiltration unit with a UM10 membrane, followed by further concentration in a Minicon B15 cell (Amicon). All fractions were extracted with 0.2 M $H_2SO_4$ (30 min on ice), and insoluble material was removed by centrifugation. Histones were precipitated from the extracts with 4 vol of 95% ethanol at −20°; the precipitates were collected by centrifugation and redissolved in distilled water.

**Polyacrylamide Gel Electrophoresis.** Histones were separated on 20-cm slab polyacrylamide gels by the system of Panyim and Chalkley (14), which gives good resolution of the acetylated components of H3 and H4. Gels contained 15% acrylamide/1.3% methylenebisacrylamide, and were run at 170 V for 30–36 hr in the cold (4°). Further resolution was achieved by running samples in a second dimension using the sodium dodecyl sulfate ($NaDodSO_4$) system of Laemmli (15) as modified by Weintraub *et al.* (16). Gel slices from the first dimension were equilibrated for 20–30 min in buffer O of O'Farrell (17) (10% glycerol/5% 2-mercaptoethanol/2.3% $NaDodSO_4$/62.5 mM Tris·HCl at pH 6.8) and then applied horizontally to the top of the $NaDodSO_4$ slab gel. The slice was sealed with melted 1% agarose in buffer O and electrophoresis was carried out at 130 V for 6 hr. Gels were stained with 0.25% Coomassie blue in methanol/acetic acid/water (5:1:5 vol/vol) and destained by diffusion in methanol/acetic acid/water (2:1:5 vol/vol).

## RESULTS AND DISCUSSION

The presence of multiply acetylated species of H4 in trout testis has been demonstrated by autoradiography of [$^{14}$C]acetate-labeled histones (18) and by isolation of acetylated peptides of H4 (19). Fig. 1 shows various stages of the DNase II/$Mg^{2+}$ fractionation procedure. The histone patterns from control chromatin (C), from the undigested pellet ($P_1$) after DNase II treatment, and from the $Mg^{2+}$-insoluble fraction ($P_2$) are indistinguishable from each other (Fig. 1). The $Mg^{2+}$-soluble fraction $S_2$, however, is greatly enriched in the diacetylated ($A_2$), triacetylated ($A_3$), and tetraacetylated ($A_4$) species of H4. The solubility of this chromatin fraction in 2 mM $Mg^{2+}$ is believed to be due to its high content of RNA (11). Treatment of this fraction with pancreatic ribonuclease resulted in precipitation of approximately 35% of the material; a sample of histones from this precipitate ($P_3$) is also shown in Fig. 1 and is likewise greatly enriched in acetylated H4 species. In this experiment, the yield of H4 in the $P_3$ fraction is low, possibly due to inefficient extraction; in other experiments the H4 pattern consistently resembled that of the $S_2$ fraction, with the addition of the RNase band. A second treatment of the supernatant to $P_3$ with RNase led to precipitation of a further 10–15% of the original digest products, and this fraction, $P_4$, resembled the $S_2$ and $P_3$ fractions in its content of acetylated H4 (Fig. 1).

The relative proportions of the acetylated H4 components at each stage of the fractionation are more readily seen in gel scans of the H4 region (Fig. 2). In whole chromatin, the monoacetylated species is most abundant, but the di- and triacetylated species are readily detected; the tetra-acetylated species is not resolved from the adjacent H2B band in these scans (Fig. 2*A*). The chromatin remaining insoluble after DNase II digestion ($P_1$) and the $Mg^{2+}$-insoluble component of the digest ($P_2$) also contain H4 with the above distribution of acetyl bands (Fig. 2 *B* and *C*). In the pellet resulting from RNase treatment of the $S_2$ fraction ($P_3$ and $P_4$, Fig. 2 *D* and *E*), the $A_2$ and $A_3$ species equal or exceed the $A_1$ in amount. $A_3$ seems to be the most prominent component in these fractions; however, as will be seen below, an unknown polypeptide migrates very close to $A_3$ in the first dimension, and the actual amount of $A_3$ present is approximately equal to the amount of $A_2$. The $A_4$ component, visible in the photograph of the original gel (e.g., Fig. 1, $P_4$), was not resolved in the scan.

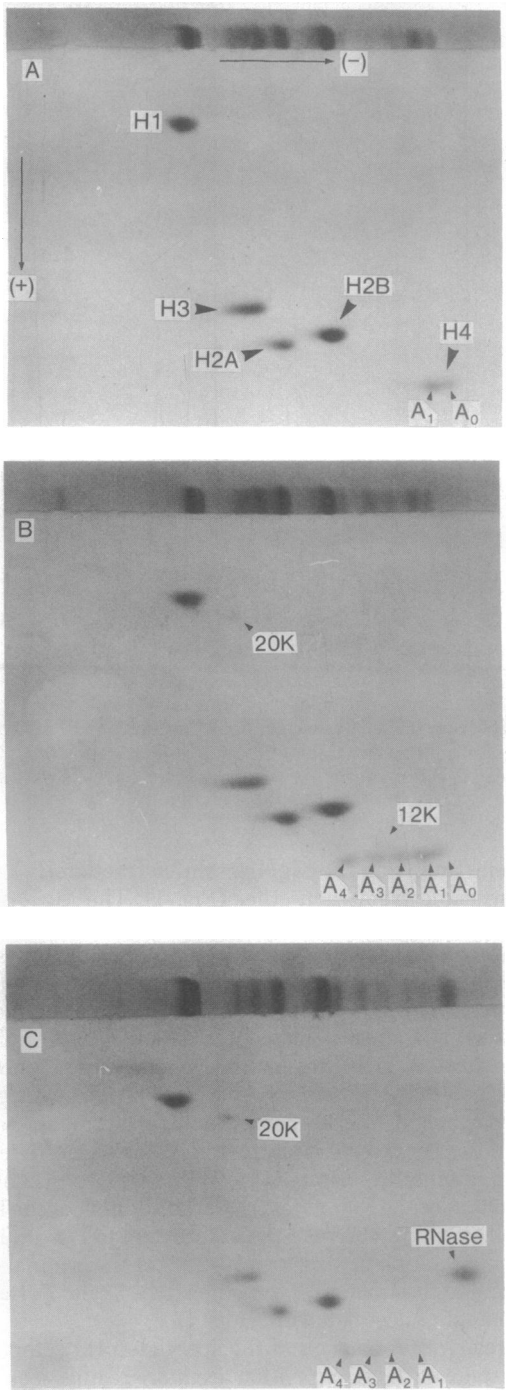


FIG. 3. Two-dimensional polyacrylamide gels of histones from chromatin fractions, stained with Coomassie blue. The first (horizontal) dimension consisted of an acid/urea gel (14), and the second (vertical) dimension consisted of the $NaDodSO_4$ system of Laemmli (15) as modified by Weintraub *et al.* (16). For the second dimension, a gel strip was excised from a lane of the acid/urea gel containing the histones; the strip was equilibrated in buffer O of O'Farrell (17) for 20–30 min, applied to the surface of the $NaDodSO_4$ slab gel, and sealed with melted 1% agarose in buffer O. The remaining region of the first-dimension strip was stained with Coomassie blue and is shown at the top of the corresponding two-dimensional separation. (*A*) Histones from control unfractionated chromatin; (*B*) histones from the $Mg^{2+}$-soluble DNase II fraction, $S_2$; (*C*) histones from the material rendered $Mg^{2+}$ insoluble by RNase digestion of $S_2$ ($P_4$). 12K and 20K are unidentified proteins. The positions of the histone fractions are shown in *A*. Components $A_0$–$A_4$ refer to subspecies of H4, as described in the legend to Fig. 1.

In order to examine the possibility that the H4 region of the gel might be contaminated with unknown protein species, we analyzed samples of histones from whole chromatin and from fractions $S_2$ and $P_4$ on two-dimensional gels in which components were separated on an acid/urea gel in the first dimension, followed by electrophoresis in a $NaDodSO_4$ gel in the second dimension. As seen in Fig. 3*A*, all histone fractions are separated from each other in this system. Examination of the $S_2$ fraction confirms that the bulk of the H4 present is in the acetylated forms (Fig. 3*B*). Furthermore, $A_4$ is now clearly visible and is present in amounts approximately equal to those of $A_3$. An unidentified component of slightly lower mobility than H4, designated 12K, migrates between $A_2$ and $A_3$, and is barely visible in the photograph of this gel (Fig. 3*B*). This component has not been detected in unfractionated chromatin. An additional unidentified component, designated 20K, coincides exactly with diacetylated H3 in the first dimension, but migrates just ahead of H1 in the $NaDodSO_4$ dimension (Fig. 3 *B* and *C*). It also seems to be associated with the template-active chromatin fraction, and on acid/urea gels gives the erroneous impression that the $A_2$ species of H3 is enriched in this fraction (see, for instance, P4 of Fig. 1, arrow). At the present time, we cannot rule out the possibility that the 12K and 20K components arise from proteolytic degradation, say by a contaminant of the DNase II. However, if this is so, they are evidently associated with the $Mg^{2+}$-soluble fraction. The absence of other low-molecular-weight species on the gels, however, argues against this possibility.

A two-dimensional gel of the $P_4$ chromatin fraction is shown in Fig. 3*C*. The lower amount of protein applied to the gel accentuates the fact that $A_3$ and $A_4$ are predominant H4 species; the $A_2$ and $A_1$ components were clearly visible on the original gel, but do not appear distinct in the photograph. The $A_0$ component was not discernible, even on the original gel. The 20K protein is present, but the 12K spot is not visible, possibly due to the lower loading.

The above results clearly show that the DNase II, $Mg^{2+}$-soluble fraction of chromatin is greatly enriched in the multiplyacetylated species of H4. Although precise quantitation of the $A_3$ and $A_4$ components is very difficult because of their low concentrations in whole chromatin, it seems likely that most of the tri- and tetra-acetylated H4 of immature trout testis is in the $Mg^{2+}$-soluble fraction, which constitutes approximately 7–10% of the total chromatin.

The $Mg^{2+}$-soluble DNase II fraction from rat liver is organized into nucleosomes and is enriched in nascent RNA and nonhistone proteins (11). It has also been shown that globin genes are enriched in the $Mg^{2+}$-soluble fraction of Friend cells which have been induced to synthesize hemoglobin (20), although there is disagreement as to whether the same enrichment occurs in uninduced cells (20, 21). However, in cells that have lost the capacity for hemoglobin induction, no enrichment of globin sequences is seen in this fraction (21).

The evidence so far thus suggests that the $Mg^{2+}$-soluble DNase II fraction is enriched in active genes sequences. Active genes are also more sensitive to digestion by DNase I (22, 23), and on this basis it was suggested that they contain nucleosomes with an altered conformation (22). The association of acetylated H4 with the template-active fraction may provide a mechanism for the proposed conformational changes, in which the presence of other specific proteins may also play a role. For instance, Levy *et al.* (24) have reported the association of two proteins, H6 and HMG-T, with active regions of chromatin. Both of these seem to be different from the 12K and 20K components reported here. It seems likely that template-active chromatin

differs in both histone conformation and in the content of nonhistone proteins from inactive chromatin.

It seems surprising that no changes in the degree of modification of the other histones, particularly H3, were observed in this study. The H3 components are not as well resolved as those of H4, however, and studies with radioactively labeled cells may help to clarify this point. The availability of a fractionation scheme in which certain modified histone species are associated with active genes should allow more detailed studies of the role of histone acetylation in transcription.

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