
Histone H4 hyperacetylation and rapid turnover of its acetyl groups in transcriptionally inactive rooster testis spermatids

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ABSTRACT

In order to study the relationship between acetylation of histones, chromatin structure and gene activity, the distribution and turnover of acetyl groups among nucleosomal core histones and the extent of histone H4 acetylation were examined in rooster testis cell nuclei at different stages of spermatogenesis. Histone H4 was the predominant acetylated histone in mature testes. Hyperacetylation of H4 and rapid turnover of its acetyl groups are not univocally correlated with transcriptional activity since they were detected in both genetically active testicular cells and genetically inactive elongated spermatids. During the transition from nucleohistone to nucleoprotamine in elongated spermatids the chromatin undergoes dramatic structural changes with exposition of binding sites on DNA (1). Hyperacetylation of H4 and rapid turnover of its acetyl groups could be correlated with the particular conformation of chromatin in elongated spermatids and might represent a necessary condition for binding of chromosomal proteins to DNA.

INTRODUCTION

Acetylation is a postsynthetic modification of the four nucleosomal core histones whose exact biological role is unknown at present. Although many observations suggest a correlation between histone acetylation and transcription(2,3), the extent of this modification in several mammalian cell types(40 % of H3 and H4) is much larger than what would be necessary if it is assumed that only about 10 % of the genome of these cells is transcribed.

A better correlation exists between transcriptional activity and the extent of hyperacetylation of H4 (4) and also with the rapid turnover of acetyl groups in a small population of histones comprising no more than 15 % of nucleosomes (5).

In order to investigate if histone H4 hyperacetylation and

the rapid turnover of its acetyl groups are univocally correlated with transcriptional activity we have determined both parameters through rooster spermatogenesis. Spermatogenesis is an excellent model to investigate the relationship between structural and functional changes of chromatin and acetylation of histones (6-9).

Chromatin undergoes dramatic changes in structure and function during rooster spermatogenesis (1,10): a) Spermatogonia, spermatocytes and early spermatids are active in nuclear transcription, whereas spermatids undergoing differentiation (spermiogenesis) and spermatozoa are inactive; b) The typical structure of active chromatin in meiotic, premeiotic and early spermatids is replaced during spermiogenesis by a more relaxed structure with exposition of binding sites on DNA and cessation of transcriptional activity; c) A massive condensation of chromatin occurs at the end of spermiogenesis when the DNA interacts with a highly basic protein, the protamine galline.

The results we report herewith concerning the presence of histone H4 hyperacetylation and rapid turnover of its acetyl groups in transcriptionally inactive elongated spermatids, support the concept that this phenomena are not univocally correlated with transcriptional activity and might represent a general mechanism for exposition of binding sites on DNA to chromosomal proteins.

MATERIALS AND METHODS

Labelling of testicular cells with ^3H -acetate.

Hubbard White Mountain roosters (25-30 weeks old) and sexually immature chickens (8-10 weeks old) were used throughout this study. Fresh rooster testes (30g) were minced with scissors and gently dissociated with a syringe in 5 volumes of Minimum Essential Medium (Eagle). The cell suspension was filtered, diluted to a final volume of 250 ml and cycloheximide was added to a final concentration of 0.2 mM. The cell suspension was cooled and sodium butyrate was added to a final concentration of 50 mM. Cells were collected by centrifugation at 2000 x g for 10 min.

Turnover of histone acetyl groups in rooster testicular cells.

Rooster testis cell suspensions were incubated with ^3H -acetate for 15 min as previously indicated. After the pulse period the cell suspension was cooled to 4°C by diluting it 10-fold with nonradioactive ice-cold medium. After centrifugation at $400 \times g$ for 20 min, the cell pellet was resuspended in fresh medium containing 1 mM sodium acetate. Aliquots of the cell suspension were incubated at 37°C for 0,5,15 and 60 min. Rooster testis cell nuclei were isolated and separated at unit gravity in the presence of 50 mM sodium butyrate, and the ^3H -acetyl content of the extracted histones from each fraction was determined as described in the following sections.

Assay for histone deacetylase activity.

The assay for enzymatic deacetylation of histone follows the method proposed by Inoue and Fujimoto (11). DNA was determined by the diphenylamine reaction (12) with calf thymus DNA as a standard. The amount of histone H4 per sample was calculated from the densitometer scan of the H4 band obtained after SDS-polyacrylamide gel electrophoresis of histones extracted from nuclei with $0.2 \text{ M H}_2\text{SO}_4$.

Isolation of rooster testis cell nuclei and separation at unit gravity.

Nuclei from rooster testis cells were isolated by the sucrose procedure previously described (10) except for the medium used (3 mM MgCl_2 , 1 mM Ca acetate, 25 mM KCl, 0.12 mM spermidine, 1 mM PMSF, 0.05 % Nonidet P-40 and 50 mM sodium butyrate).

Purified nuclei were separated by sedimentation at unit gravity (10). All media used for the separation of nuclei contained 1 mM PMSF and 50 mM sodium butyrate. Five different fractions of nuclei were obtained: tetraploid primary spermatocytes (stage I); small primary spermatocytes, secondary spermatocytes and spermatogonia cells (stage II); round and elongating spermatids (stage III); elongated spermatids and testicular spermatozoa (stage IV).

Isolation and analysis of acetylated histones.

Histones were extracted in $0.2 \text{ M H}_2\text{SO}_4$ and precipitated with 5 vol of ethanol. Electrophoresis was performed either

in sodium dodecyl sulfate exponential polyacrylamide gels (10 %-16 %) as described by O'Farrell (13) or in acetic acid/urea/Triton X-100/polyacrylamide gels containing 7.5 M urea and 6 mM Triton X-100 as described by Zweidler (14). Gels were stained either with amido black or Coomassie blue and they were scanned with a model 2410 Gilford linear transport scanner at 600 nm. The methods of Bonner and Laskey (15) and Laskey and Mills (16) were used to prepare gels for fluorography of the ^3H -labelled histone. Presensitized Fuji X-Ray film was exposed at -70°C for 15 days.

RESULTS

Acetylation of histone H4 in immature and mature whole testes.

Electrophoretic analysis in sodium dodecyl sulfate polyacrylamide gels of histones extracted from immature chicken testes, enriched in spermatogonia and spermatocytes, and from mature testes, enriched in spermatids and spermatozoa, showed that the highest degree of acetylation of nucleosomal core histones is present in histone H4 derived from mature testes (Fig. 1).

Electrophoretic analysis of histones in acetic acid/urea/

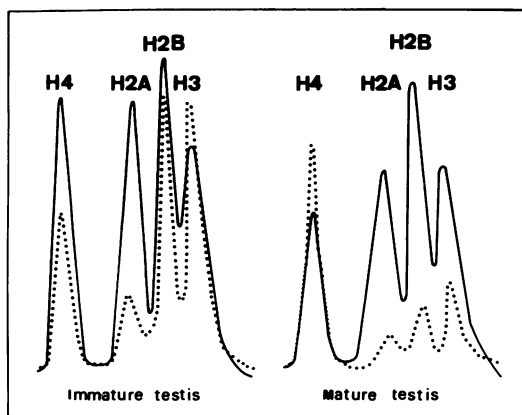


Figure 1: Acetylation of nucleosomal core histones in immature and mature chicken testes. Testicular cells were incubated in the presence of ^3H -acetate as described in the experimental section. Histones were extracted from purified nuclei and electrophoresed in SDS-polyacrylamide gels. (—) Densitometry of the gels stained with amido black. (.....) Densitometry of the fluorogram.

Triton X-100 polyacrylamide gels resolved five H4 subspecies containing none ($H4ac_0$), one ($H4ac_1$), two ($H4ac_2$), three ($H4ac_3$) or four ($H4ac_4$) acetylated lysine residues. Fluorograms of these gels showed that under the conditions of labelling, 3H -hyperacetylated forms of histone H4 are more abundant in mature than in immature testes (Fig. 2).

Acetylation of histone H4 in rooster testis cell nuclei separated by sedimentation at unit gravity.

Electrophoretic analysis in sodium dodecyl sulfate polyacrylamide gels of the acid-soluble nuclear proteins extracted from rooster testis cell nuclei separated by sedimentation at unit gravity, showed that all core nucleosomal histones, but not histone H1, were acetylated at different stages of spermatogenesis (Fig. 3A). The specific activity of 3H -acetylated histone H4 increases during spermiogenesis reaching its maximum level in transcriptionally inactive elongated spermatids (stage IV nuclei) as it is shown in Fig. 3B.

Electrophoretic analysis in acetic acid/urea/Triton X-100/polyacrylamide gels showed that the specific activity of tetra-acetylated H4 increases during spermatogenesis reaching its

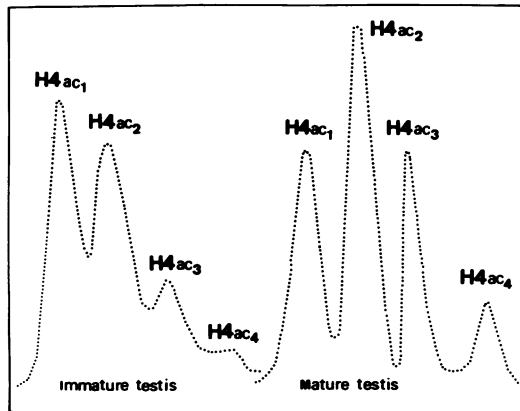


Figure 2: Distribution of 3H -acetate among histone H4 subspecies in immature and mature chicken testes. Testicular cells were incubated in the presence of 3H -acetate as described in the text. Histones were extracted from purified nuclei and electrophoresed in acetic acid/urea/Triton X-100/polyacrylamide gels. (.....) Densitometer scans of the fluorogram of acetylated H4 subspecies containing one ($H4ac_1$), two ($H4ac_2$), three ($H4ac_3$) and four ($H4ac_4$) acetylated lysine residues.

maximum level in transcriptionally inactive stage IV nuclei (fig. 4). The specific activity of diacetylated H4 is higher in dividing testicular cells (stages I and II) than in non-dividing spermatids (stages III and IV). The specific activity of threeacetylated H4 could not be determined in these gels because a nuclear spermatidal protein (M. Chiva, unpublished data) migrated with similar electrophoretic mobility to the threeacetylated H4 molecule (Fig. 4A).

The distribution of ^3H -acetate among histone H4 subspecies (H4ac_1 , H4ac_2 , H4ac_3 and H4ac_4) determined by fluorography showed that the ^3H -tetra and ^3H -threeacetylated forms of histone H4 are more abundant in transcriptionally inactive stage IV nuclei than in transcriptionally active stages I and II, whereas the opposite is true for ^3H -mono and ^3H -diacetylated histone H4 subspecies (Fig. 5). The nuclear spermatidal protein migrated behind histone H3 in SDS-polyacrylamide gels.

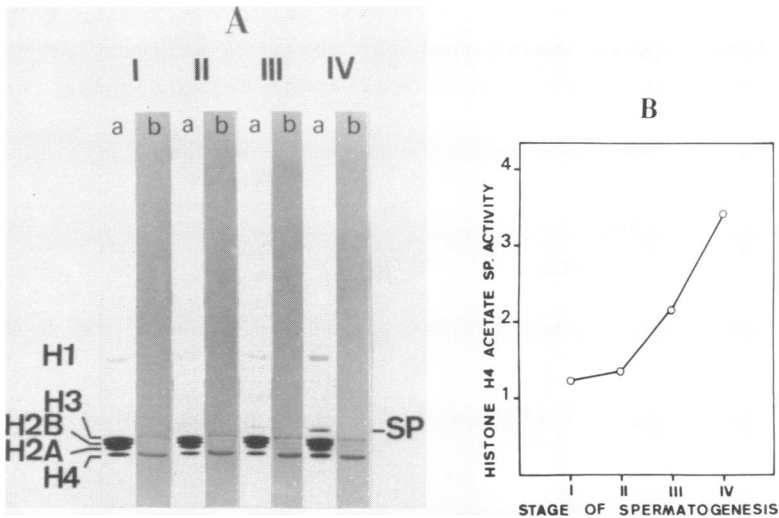


Figure 3: Acetylation of histone H4 through rooster spermatogenesis. Histones were extracted from nuclei separated by sedimentation at unit gravity. (A) SDS-polyacrylamide gel electrophoresis of 3H-acetylated histones. (a) Gel stained with amido black (b) fluorogram of the gel. (B) Specific activity of 3H-acetylated H4 at different stages of rooster spermatogenesis calculated from the densitometer scans of the H4 bands shown in A. (I) Tetraploid primary spermatocytes, (II) small primary spermatocytes, secondary spermatocytes and spermatogonia, (III) early spermatids, (IV) elongated spermatids.

The fluorograms of these gels showed that the spermatidal protein was not acetylated (Fig. 3A). Therefore, at the concentration loaded in the acetic acid/urea/Triton X-100/polyacrylamide gels, the spermatidal protein cannot contribute to the band intensity of ^3H -threacetylated H4 in the fluorogram. Turnover of histone acetate in rooster testis cells at different stages of spermatogenesis.

When testicular cells were incubated for 15 min with ^3H -acetate and the pulse was followed by a chase with a medium containing nonradioactive acetate, the ^3H -acetate label of nucleosomal core histones decreased rapidly both in transcriptionally active cells (stages I and II) and in transcription-

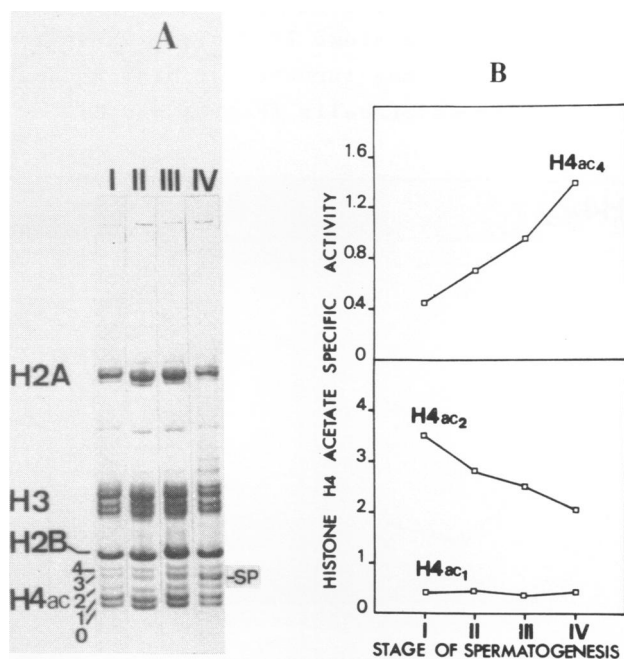


Figure 4: Acetylation of histone H4 through rooster spermatogenesis. Histones were extracted from nuclei separated by sedimentation at unit gravity. (A) Acetic acid/urea/Triton X-100/polyacrylamide gel electrophoresis of ^3H -acetylated histones stained with Coomassie blue. (B) Specific activity of ^3H -acetylated H4 subspecies at different stages of rooster spermatogenesis calculated from the densitometer scans of the stained gel shown in A and its fluorogram. The specific activity of threacetylated H4 could not be determined because a specific nuclear spermatidal protein (SP) migrated with similar electrophoretic mobility to H4_{ac3}. I, II, III and IV as in Fig. 3.

ally inactive cells (stage IV) as it is shown in Fig. 6A. A high turnover of histone H4 acetate has also been detected in both types of cells (Fig. 6B).

Histone deacetylase activity in nuclei at different stages of spermatogenesis.

In order to explore if the rapid turnover of histone acetate could be correlated with histone deacetylase activity, this enzymatic activity was determined in rooster testis cell nuclei at different stages of spermatogenesis. In the range of DNA concentrations used (50-150 µg of DNA per assay) histone deacetylase activity expressed as cpm of ³H-acetate released/30 min/H4 content per assay, was 3150 for a fraction containing transcriptionally active nuclei (stages I and II) and 4047 for transcriptionally inactive stage IV nuclei. These results are in good correlation with the turnover of histone acetate determined in both types of cells (Fig. 6A and B).

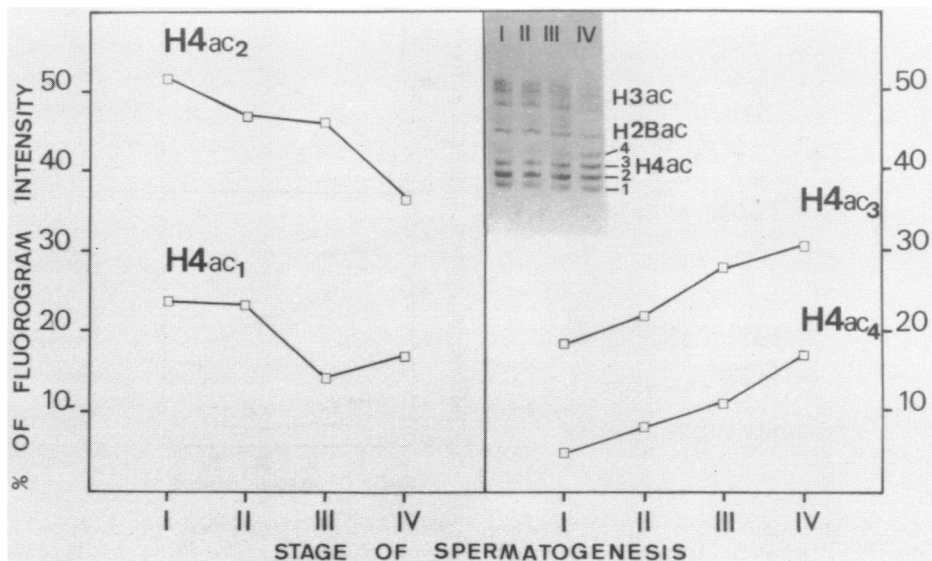


Figure 5: Distribution of ³H-acetate among histone H4 subspecies through rooster spermatogenesis. Histones were extracted from purified rooster testis cell nuclei separated by sedimentation at unit gravity and electrophoresed in acetic acid/urea/Triton X-100/polyacrylamide gels. The percentage of each acetylated H4 subspecies was calculated from the densitometer scans of the fluorogram shown in the insert of this figure. I, II, III and IV as in Fig. 3.

DISCUSSION

The results presented in this paper indicate that hyperacetylation of histone H⁴ and rapid turnover of its acetyl groups occur in genetically active meiotic and premeiotic cells as well as in genetically inactive elongated spermatids.

Acetylation of lysine residues in the basic domain of histone H⁴ might rapidly and reversibly expose binding sites on DNA for interaction with chromosomal proteins such as enzymes and regulatory proteins involved in transcription (2,3). Acetylation of histones in genetically active rooster testis cells fits with this model.

A higher specific activity of diacetylated histone H⁴ was detected in dividing testicular cells (stages I and II) than in non-dividing spermatids (stages III and IV, Fig. 5). This

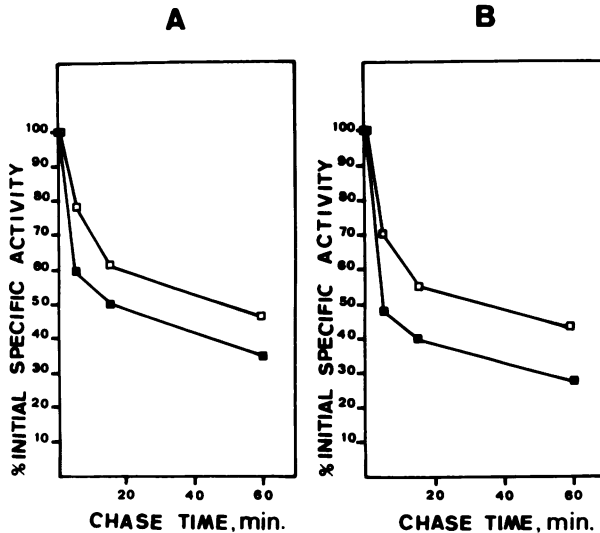


Figure 6: Turnover of ³H-acetate associated with the core histones (A) and histone H⁴ (B) during rooster spermatogenesis. Testicular cells were labelled with ³H-acetate for 15 min, as described in the text. Cells were cooled and resuspended in fresh medium containing 1 mM sodium acetate. The zero time sample was taken and aliquots of the cell suspension were incubated at 37°C for 5, 15 and 60 min. Cell nuclei were purified from each fraction and separated at unit gravity in the presence of 50 mM sodium butyrate. The specific activity of the ³H-acetylated histones was determined by solubilization of whole histone in NCS (Amersham) and by densitometry of the stained SDS-gels and fluorograms. (□-□-□) Transcriptionally active nuclei (stages I and II). (■-■-■) Transcriptionally inactive nuclei (stage IV).

result is in accordance with the observation of a peak of diacetylated H₄ at S-phase in *Physarum polycephalum* and HTC cells (4,5).

Candido and Dixon (6) first proposed that histone acetylation in genetically inactive trout spermatids could be involved in the replacement of somatic histones by protamine. Our data do not support the idea that histone acetylation is a general mechanism for histone removal, because in mature rooster testis only histone H₄ was highly acetylated (Fig.1).

Christensen and Dixon (17) recently proposed that histone H₄ hyperacetylation in trout spermatids could be a mechanism for exposition of DNA binding sites to protamine. However such exposition of binding sites has not been demonstrated during trout spermiogenesis.

A clear correlation exists between hyperacetylation of histone H₄ in rooster transcriptionally inactive spermatids (nuclei of stage IV, Figs. 3,4 and 5) and exposition of DNA binding sites (1,10) in the chromatin of these cells. The exposition of DNA binding sites in chromatin obtained from transcriptionally inactive stage IV nuclei, was demonstrated measuring the following parameters: 1) Binding of actinomycin D, 2) Number of RNA initiation sites for *E. coli* RNA polymerase, 3) Rate of RNA chain elongation and 4) Kinetics of formation of stable binary complexes between RNA polymerase and chromatin.

We wanted to know whether acetylation of histones in transcriptionally active and transcriptionally inactive cells was metabolically stable or unstable. Our results proved the existence of a rapid turnover of histone acetate both in transcriptionally active cells (stages I and II) and in transcriptionally inactive cells (stage IV) as it is shown in Fig.6. Moreover, histone deacetylase activity assayed in nuclei correlated with histone acetate turnover in both types of cells.

From the present results we postulate that, with independence of the transcriptional activity of the cell, through hyperacetylation of histone H₄ and rapid turnover of its acetyl groups, sites on DNA can be rapidly and reversibly exposed, facilitating the binding of chromosomal proteins to DNA. The functional consequences of histone H₄ hyperacetylation

will depend on the specific proteins bound to the DNA at each particular stage of the cell differentiation process.

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