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# Immunochemical detection of changes in chromatin subunits induced by histone H4 acetylation

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**Native, reassociated, and reconstituted core particles from chicken erythrocytes were compared by both biophysical and immunochemical methods. No significant difference between the three types of core particles could be demonstrated by electron microscopy, circular dichroism, or immunochemical analysis with antisera to histone H2B, H2A, and H3. Core particles were also reconstituted with calf thymus non-acetylated H3, H2A, and H2B with either mono-, di-, or tri-acetylated H4 isolated from cuttle-fish testes. The hyperacetylation of H4 did not significantly alter the biophysical characteristics of core particles but it induced several changes in their immunochemical reactivity. Binding to core particles of antibodies specific for H2A, H3, and for the IRGERA (synthetic C-terminal) peptide of H3 was considerably decreased when di- or tri-acetylated H4 was used for reconstitution, whereas binding of H2B antibodies remained the same. Our results suggest that the presence of hyperacetylated H4 within core particles leads to conformational changes that alter the antigenic determinants of several of the histones present at the surface of chromatin subunits. Since histone acetylation is correlated with the open structure of active chromatin, it may become possible to monitor the activity of chromatin by immunochemical methods.**

*Key words:* histone acetylation/chromatin/immunochemistry/antibodies to histones

## Introduction

Antibodies to histones represent one of the most specific probes for determining the accessiblity of histones at the surface of chromatin. Only a few epitopes (antigenic determinants) of histones H2B, H3, and H5 have been localized in the primary structure of these proteins (Absolom and Van Regenmortel, 1977, 1978; Mura *et al.*, 1980) and only one epitope each of H2B and H3 has been found to be expressed at the periphery of the nucleosome (Absolom and Van Regenmortel, 1978; Muller *et al.*, 1982). In spite of the limited amount of information concerning the molecular structure of epitopes found at the surface of chromatin subunits, antibodies to histones are nevertheless valuable tools for analysing fine details of the chromatin surface. Antibodies to globular proteins recognize epitopes made up of a small patch of 5-7 amino acid residues that are kept in a particular conformation by the folding of the polypeptide chain; the residues comprising the epitope may be contiguous in the sequence or may only be juxtaposed in space as a result of chain folding (Sela, 1969; Arnon and Geiger, 1977; Atassi and Smith, 1978). In macromolecular assemblies such as nucleosomes it is also conceivable that some epitopes arise from the juxtaposition of residues from separate peptide chains. Furthermore, bonds formed between the core histones are likely to lead to conformational changes in the constituent molecules which may alter the epitopes. For these various reasons antibodies to histones may be expected to be sensitive tools for detecting any conformational changes that may occur at the surface of chromatin as a result of changes in histone-histone or DNA-histone interactions (Romac *et al.*, 1981).

We have used histone antibodies to probe for any antigenic differences between native, reassociated, and reconstituted core particles from chicken erythrocytes. We have also compared reconstituted core particles that contained the usual histone H4 (i.e., ~90% non-acetylated) with core particles containing either mono-, di-, or tri-acetylated H4. Although these various particles appeared to be identical when analysed by electron microscopy and circular dichroism (c.d.), the core particles that contained tri-acetylated H4 showed considerable antigenic changes when tested with different histone antisera. It seems that the presence of hyperacetylated H4 within core particles leads to a conformational change that alters the accessibility or nature of the epitopes of several of the histones present at the surface of chromatin subunits.

It is generally assumed that post-synthetic histone acetylation is one of the mechanisms that produce the open configuration of active chromatin. In hyperacetylated H4, the acetylation of lysine residues 5, 8, 12, and 16 leads to a less positively charged N-terminal region which is correlated with a weakening of histone-DNA interaction, an expansion of $\alpha$-helical regions of histones (Bode *et al.*, 1980), and an increased sensitivity towards DNase I (Vidali *et al.*, 1978; Nelson *et al.*, 1978; Simpson, 1978). The conformational changes are believed to cause partial nucleosome disassembly and to allow RNA polymerase read-through. Although the exact sequence of events linking histone acetylation to the promotion of transcription is not yet fully elucidated (Mathis *et al.*, 1980), it is clear that acetylated chromatin subunits possess an altered and more open conformation than that of inactive chromatin. The data we present demonstrate that this altered conformation can also be monitored by immunochemical methods.

## Results

### *Analysis of non-acetylated reassociated and reconstituted core particles*

Native chicken erythrocyte core particles prepared as previously described (de Murcia *et al.*, 1980) were characterized by their c.d. spectrum and by electron microscopy. As shown in Figure 1, the maximum ellipticity value $|\theta|_{max}$ at 283.5 nm was 1300 degrees/cm$^2$/dmol (± 150) and the minimum value $|\theta|_{min}$ at 294.5 nm was −590 (± 150), indicating a correct DNA folding (de Murcia *et al.*, 1980). This spectrum indicates the total absence of any unfolded core particles since the presence of non-constrained DNA would have led to

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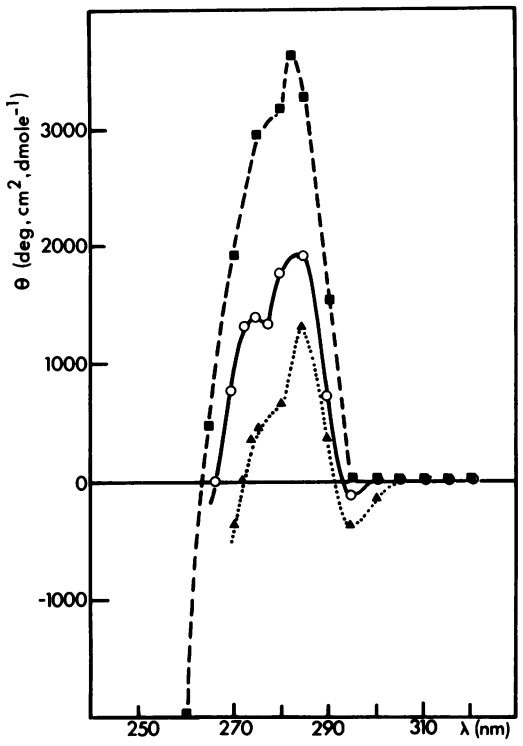


**Fig. 1.** C.d. spectra of native chicken erythrocyte core particles (▲——▲); core particles reconstituted with non-acetylated chicken erythrocytes core histones (○——○); core particles reconstituted with non-acetylated calf thymus H2B, H2A, H3, and acetylated cuttle-fish testis H4 (■——■). Modified H4 of different degrees of acetylation were used. Data are expressed as molecular ellipticity per dmol of DNA phosphate (degrees/cm²/dmol).

much higher ellipticity values. In the electron microscope native core particles appeared as totally folded particles (Figure 2A). When native core particles were rapidly dissociated in 2 M NaCl and immediately reassociated by a one-step decrease in ionic strength, the resulting reassociated core particles displayed the same c.d. spectrum and microscopic appearance as the native core particles (data not shown).

Reconstituted core particles were prepared by mixing native core particle DNA (146 bp) with stoichiometric amounts of the four isolated chicken erythrocyte core histones. The c.d. spectrum of these reconstituted particles indicated a shift of ellipticity to slightly higher values (Figure 1). On the other hand, electron microscopy of this preparation showed a high proportion of native-like core particles with only a small quantity of free DNA (Figure 2B). It is this free DNA which is responsible for the increase in c.d. values. Correlated results from c.d. spectroscopy and electron microscopy indicated that the reconstitution yield was ~90% (± 2%).

The correctness of assembly within the reassociated and reconstituted core particles was also tested by measuring the comparative binding of various histone antisera to the native, reassociated, and reconstituted core particles. This study was performed using a solid-phase radioimmunoassay (RIA). As shown in Figure 3A, the native core particles reacted with antibodies to H2B, H2A, H3, and to the IRGERA peptide (synthetic C-terminal hexapeptide of H3) but not with antibodies to H4. The inability of H4 antibodies to react with native core particles was verified with three H4 antisera

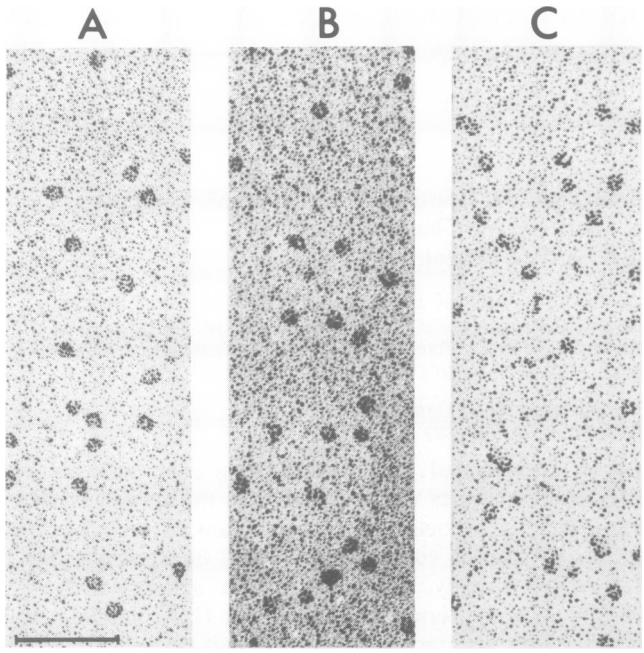


**Fig. 2.** Electron microscopy of native chicken erythrocyte core particles (**A**); core particles reconstituted with non-acetylated core histones (**B**); core particles reconstituted with acetylated H4 (**C**). The preparations are the same as those used for obtaining Figure 1. The bar = 1000 Å.

prepared in different rabbits. The binding of the different histone antisera to the reassociated and reconstituted core particles was roughly similar to that observed with native core particles (Figure 3B and C) although in some experiments with H2B antisera it was 20% lower in reconstituted particles. In separate experiments (not shown in Figure 3C), H2A antisera were also found to react with reconstituted core particles (see Figure 5). Although the extent of antibody binding varied somewhat in different reconstitution experiments, the results obtained with the three types of core particles (native, reassociated, and reconstituted) were sufficiently similar to warrant the conclusion that the reassociation and reconstitution procedures did not produce a major rearrangement of histone epitopes at the surface of the chromatin subunits.

*Analysis of acetylated core particles*

To study the influence of histone acetylation on the structure of core particles, reconstitution experiments were performed with native core particle DNA and non-acetylated calf thymus H2A, H2B, and H3 plus H4 of different degrees of acetylation ($Ac_1H4$, $Ac_2H4$, and $Ac_3H4$). The various acetylated forms of H4 were isolated from cuttle-fish testis chromatin in which high levels of natural acetylation are present (Wouters-Tyrou *et al.*, 1981). The purity of the mono-, di-, and tri-acetylated forms of H4 was controlled by urea-polyacrylamide gel electrophoresis (Figure 4). The reconstitution yield was evaluated by counting several thousand core particles and free DNA molecules present on electron micrographs of reconstituted material and was found to be 67 ± 2% in all three cases of mono-, di-, and tri-acetylated core particles. This somewhat low reconstitution yield is probably due to some denaturation of H4 during the purification procedure, but is obviously not linked to the degree of acetylation of H4. The reconstituted core particles containing acetylated H4 were found to be correctly folded as judged by electron microscopy (Figure 2C); their molar ellipticity $|\theta|_{max}$

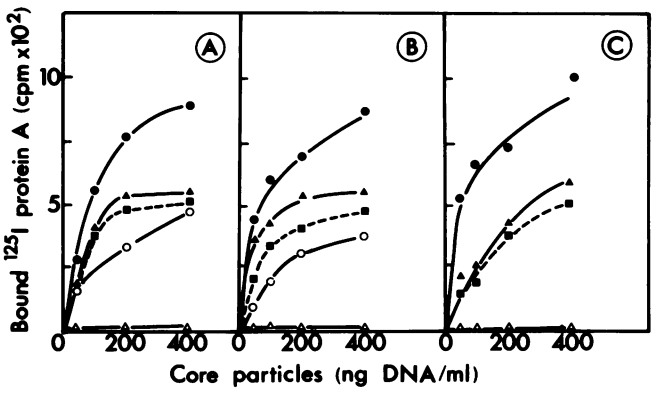


**Fig. 3.** Binding of various histone antisera (diluted 1:100) in RIA to increasing quantities of native chicken erythrocyte core particles (**A**); reassociated core particles (**B**); and reconstituted core particles (**C**). Antisera used: anti H2B (●——●), anti H3 (▲——▲), anti IRGERA (■——■), anti H2A (○——○), anti H4 (△——△). Binding of normal rabbit serum to the various preparations was insignificant.

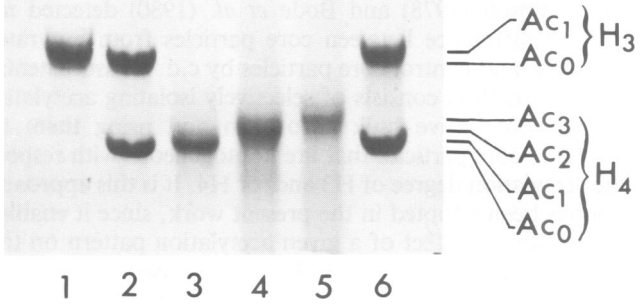


**Fig. 4.** Polyacrylamide gel electrophoresis analysis of the H3 and H4 used in core particles reconstituted experiments. (**1**) Control non-acetylated calf thymus H3; (**2**) calf thymus non-acetylated (H3-H4) complex; (**3**) mono-acetylated cuttle-fish testis H4; (**4**) di-acetylated cuttle-fish testis H4; (**5**) tri-acetylated cuttle-fish testis H4; (**6**) mono-acetylated calf thymus (H3-H4) complex. Electrophoresis was performed in 2.5 M urea (Panyim and Chalkley, 1969). The differently acetylated forms of H4 and H3 are indicated $Ac_0$ to $Ac_3$.

at 283.5 nm was found to be 3600 (± 150) degrees/cm²/dmol (Figure 1). This value is consistent with the presence of 67% properly folded core particles ($\theta$ = 1300) and 33% free DNA ($\theta$ = 8000). The remaining 33% of free histones (in aggregated state) had been eliminated by centrifugation. It seems, therefore, that the hyperacetylation of H4 does not lead to a major alteration of the biophysical characteristics of the core particles.

The influence of H4 acetylation on the immunochemical reactivity of the reconstituted core particles was tested with the histone antisera described above. Several binding curves obtained with particles showing different degrees of H4

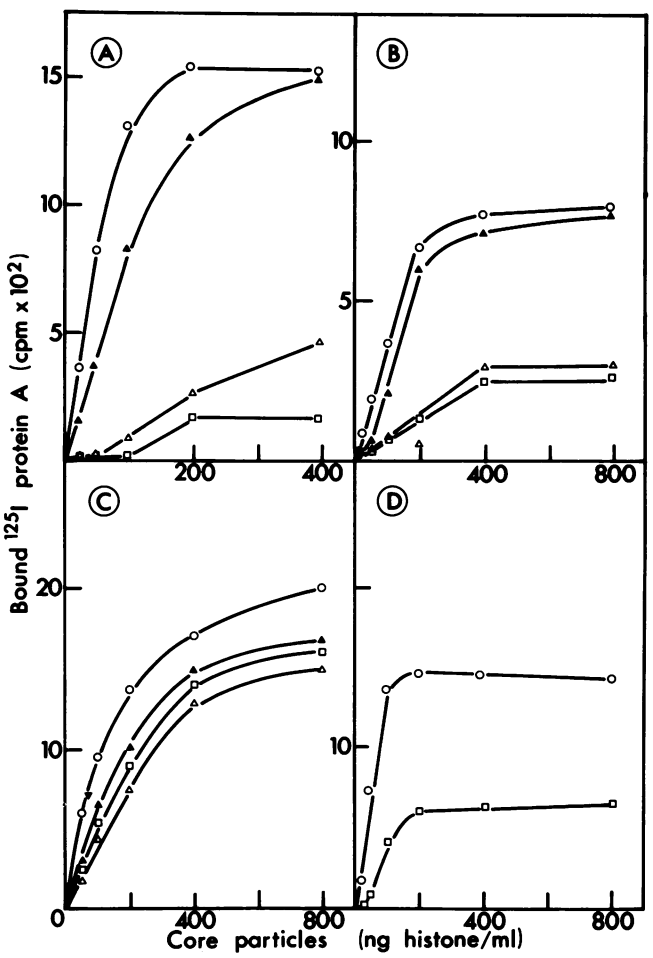


**Fig. 5.** Binding of various antisera (diluted 1:100) in RIA to increasing quantities of reconstituted chicken erythrocyte core particles (○——○) and reconstituted core particles containing the three calf thymus histones H3, H2A, H2B, and different forms of cuttle-fish H4: mono-acetylated form (▲——▲), di-acetylated form (△——△), or tri-acetylated form (□—□). Antisera used: anti H3 (**A**), anti IRGERA (**B**), anti H2B (**C**), anti H2A (**D**). Binding of normal rabbit serum was insignificant in all cases. The histone concentration was determined by assuming a histone/DNA ratio of 1.2 for the core particle and taking the reconstitution yield into account.

acetylation are presented in Figure 5 and the comparative binding percentages obtained in several experiments are summarized in Table I. The interaction between H3 and its specific antibodies decreased when the degree of acetylation of H4 increased from 0 to 3 (Figure 5A), the major effect being observed in the change from $Ac_1H4$ to $Ac_2H4$. When IRGERA antiserum was used, the same phenomenon was observed (Figure 5B). This means that increasing acetylation of H4 diminishes the accessibility of the C-terminal end of H3 at the surface of the core particle. Likewise, the accessibility of H2A to specific antibodies was also greatly decreased when tri-acetylated H4 was incorporated into core particles (Figure 5D) since a 56% decrease in antibody binding was observed (Table I). The binding of H2B antibodies (Figure 5C) seemed to be the least affected since only ~25% decrease in binding occurred and this was not dependent on the degree of H4 acetylation (Table I). This last result also demonstrates that the three populations of acetylated core particles bind equally well to the solid-phase in the RIA test, and confirms that the measured decrease in antibody binding is not due to an artefactual variation of antigen concentration in the test.

**Table I.** Comparative binding of histone antisera to core particles reconstituted with non-acetylated and acetylated H4 ($Ac_0$ to $Ac_3$) measured by RIA

| Histones present in reconstituted core particles[a]: H3, H2B, H2A, and | Antisera[b] anti H3 | anti IRGERA[c] | anti H2A | anti H2B |
|---|---|---|---|---|
| $Ac_0$ H4 | 100% | 100% | 100% | 100% |
| $Ac_1$ H4 | 59–98[d] | 47–100 | – | 74–86 |
| $Ac_2$ H4 | 17–32 | 23–41 | – | 62–79 |
| $Ac_3$ H4 | 10–12 | 16–32 | 44 | 72–86 |

[a]Core particles used at 400 ng histone/ml.
[b]Antisera were diluted 1:100.
[c]IRGERA: C-terminal hexapeptide of histone H3 (see Muller *et al.*, 1982).
[d]Range of percentage values obtained in three separate experiments.

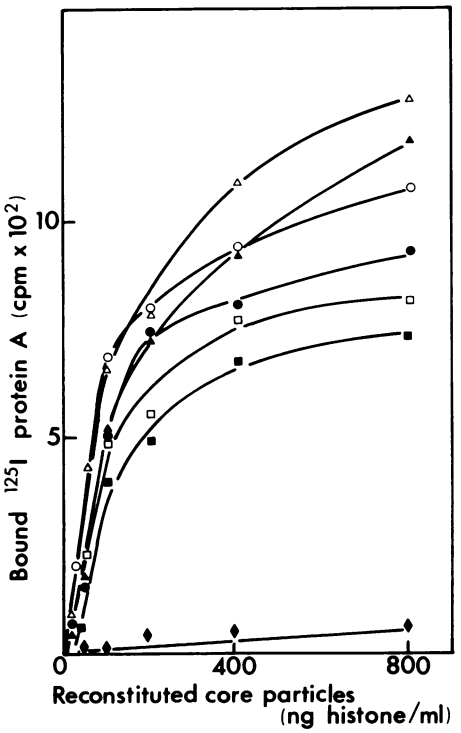


**Fig. 6.** Binding of various histone antisera (diluted 1:100) in RIA with increasing quantities of reconstituted core particles containing calf thymus H2B, H2A, and non-acetylated (closed symbols) or mono-acetylated (open symbols) H3-H4 complex. Antisera used: anti H2B (▲), anti H3 (●), anti IRGERA (■), normal rabbit serum (♦).

In a second series of experiments, core particles were reconstituted with calf thymus core histones H2A and H2B and non-acetylated or mono-acetylated H3 and H4 extracted as a complex (see Figure 4). In both cases, the reconstitution yield was ~90% and the core particles were correctly folded as shown by c.d. analysis and electron microscopy (data not shown). There was no significant difference in the binding of the various histone antisera to core particles with incorporated non-acetylated or mono-acetylated H3 and H4 (Figure 6). Although the presence of mono-acetylated H3 and H4 introduces the same number of acetyl groups in the core particles as when the diacetyl H4 is used for reconstitution (four acetyl groups per core particle), the reactivity of the mono-acetylated H3-H4 core particles with respect to the H3 and IRGERA antibodies was not altered (compare Figures 5A and B with Figure 6). It seems that the location of the acetylation sites is more critical than the total number of acetyl groups introduced in the core particles.

## Discussion

Among the possible histone modifications, it is chiefly acetylation that has been invoked as an activation mechanism of chromatin at the level of core particles. Acetylation is found in the N-terminal part of the core histones (De Lange *et al.*, 1975) and is likely to loosen DNA-histone interaction, thus offering an increased accessiblity to RNA polymerase (Nelson *et al.*, 1980). Several workers have emphasized the correlation between an enhancement of histone acetylation and hypersensitivity to DNase I (Vidali *et al.*, 1978; Nelson *et al.*, 1978), and it is well-known that active chromatin regions show such a pattern of DNase I hypersensitivity (Weintraub *et al.*, 1976; Garel *et al.*, 1976). Furthermore, this modification is generally transient (Jackson *et al.*, 1975; Cousens *et al.*, 1979), which is a prerequisite to a dynamic process such as transcription.

The level of acetylation found in bulk chromatin and in preparations of core particles is normally rather low. At least two approaches have been used to obtain more highly acetylated core particles. One method consists of increasing the acetylation level of chromatin by growing cells in the presence of butyrate (Ripp *et al.*, 1977) and then extracting hyperacetylated core particles that are modified at the proper *in vivo* sites of H3 and H4. It has been shown that butyrate is a non-competitive inhibitor of deacetylases (Perry *et al.*, 1979). Simpson (1978) and Bode *et al.* (1980) detected no noticeable difference between core particles from butyrate-treated cells and control core particles by c.d. measurements.

Another method consists of selectively isolating acetylated histones from native bulk chromatin and using them to reconstitute core particles that are homogeneous with respect to the acetylation degree of H3 and/or H4. It is this approach which has been adopted in the present work, since it enables one to study the effect of a given acetylation pattern on the configuration of core particles. The $Ac_3H4$ used in this work was known to be acetylated at lysines 5, 12, and 16 (Wouters-Tyrou *et al.*, 1981). Our c.d. measurements showed no difference between various types of acetylated reconstituted core particles and non-acetylated ones, which means that no drastic structural modification had occurred upon acetylation. This conclusion is consistent with the similar appearance of all core particles in the electron microscope (Figure 2).

The use of antibodies for measuring the exposure of histone epitopes in core particles represents another sensitive method for determining the correctness of assembly in reconstituted core particles. Using antisera to the core histones, we found that the introduction of acetylated H4 into core particles led to several changes in their immunochemical reactivity. Binding to core particles of antibodies specific for H3 and for the IRGERA peptide of H3 was considerably reduced when $Ac_2H4$ or $Ac_3H4$ were used for reconstitution. It is known that the C-terminal IRGERA sequence of H3 is not involved in complex formation and is mobile and immunoreactive in free histone and chromatin (Moss *et al.*, 1976; Morris and Lewis, 1977; Böhm *et al.*, 1977, 1981; Muller *et al.*, 1982). Since in the $Ac_3H4$ used for

reconstitution the lysines are modified at positions 5, 12, and 16 (Wouters-Tyrou *et al.*, 1981), the resulting local decrease in positive charge probably induces in the core particle a conformational change that sterically hinders the binding of IRGERA antibodies. This result is consistent with the core particle model of Ohlenbusch (1979, 1981) that is based on an antiparallel alignment of histone homodimers and in which the N-terminal region of H4 and the C-terminal part of H3 are close neighbours. The fact that the reactivity of IRGERA antibodies was not affected when both mono-acetylated H3 and H4 were incorporated into core particles also supports this model, since it proposes that the acetylated N-terminal regions of H3 and H4 are at opposite ends of the particle (Ohlenbusch, 1981).

The conformational change induced by the presence of acetylated H4 in the core particle also caused a major decrease in the binding of H2A antibodies but had less influence on the binding of H2B antibodies. In the latter case, the binding to core particles was only reduced by ~25% and it made no difference whether H4 was mono-, di-, or tri-acetylated (Table I). The smaller effect of H4 acetylation on the binding of H2B antibodies may simply reflect the fact that histone H2B is the most accessible and immunoreactive histone in core particles (Bustin, 1979; Absolom and Van Regenmortel, 1978). In the case of H3, which is one of the least accessible histones in chromatin, it was found that specific H3 and IRGERA antibodies showed the largest drop in binding activity to acetylated core particles.

Furthermore, the immunochemical reactivity of H3 was progressively decreased when H4 of increasing degree of acetylation was incorporated into core particles. It is known that the conformational changes in chromatin induced by H4 acetylation are more pronounced when more lysine residues are modified; this finding is in agreement with the view that structural changes involved in the promotion of trancription are correlated with the presence of the most highly acetylated histones (Chahal *et al.*, 1980). Although other factors such as absence of H1, presence of HMG proteins, or presence of histone variants may also be required for promoting transcription, it is clear that the reversible acetylation of histones is correlated to the open structure of active chromatin. Acetylation of H4 is minimal at prophase and metaphase when chromosomes are highly condensed and little RNA synthesis is taking place, while it is maximal in mid S-phase and mid G2 phase (Chahal *et al.*, 1980).

Our results indicate that conformational changes induced by acetylation can be monitored with histone antibodies, and thus open the way to the development of immunochemical probes for identifying various stages of the cell cycle. If antibodies specific for the active configuration of chromatin components could be prepared, it may become possible to isolate active chromatin regions by immunochemical means.

## Materials and methods

*Preparation of core particles*

Chicken erythrocyte native core particles were prepared and characterized as previously described (de Murcia *et al.*, 1980). Reassociated core particles were prepared from native core particles as follows: core particles were suspended, at a concentration of 2 mg DNA/ml, in buffer 'R' containing 20 mM Tris pH 7.4 and 1 mM EDTA, then dissociated by the addition of an equivalent volume of buffer R-4 M NaCl and finally reassociated by direct dilution of the histone-DNA mixture from 2 M to 0.25 M NaCl with buffer R.

Reconstituted core particles were made with native core particle DNA (146 bp) and stoichiometric amounts of the four isolated core histones. In a first step, the four core histones, at a concentration of 2.5 mg/ml, were dissolved together in a medium containing 6 M urea, 4% v/v 2-mercaptoethanol, 0.01 M HCl, and left at room temperature for 12 h. The core histone solution was then dialysed against buffer R-2 M NaCl, 5 mM dithiothreitol and mixed with a core particle DNA solution (2 mg/ml) in buffer R-2 M NaCl. The ionic strength of this mixture was decreased by direct dilution with buffer R from 2 M to 0.25 M NaCl. When required, turbid solutions were clarified by centrifugation (20 min, 47 000 *g*); this treatment did not produce a significant decrease of o.d. 260 nm.

*Preparation of histones*

Chicken erythrocyte and calf thymus core histones, H2A, H2B, H3, and H4, were either acid-extracted and purified by chromatography (Johns, 1964) or purified by the saline method of Van der Westhuyzen and Von Holt (1971). The purity of the proteins was assessed by electrophoresis on 18% polyacrylamide-SDS gel. Calf thymus non-acetylated or mono-acetylated H3 and H4 were isolated as equimolecular complexes (H3-H4) by ion-exchange chromatography on Bio-Rex 70 (Bio Rad) of an equimolecular mixture of pure H3 and H4 histones (Couppez *et al.*, in preparation). The di- and tri-acetylated forms of H4, which are naturally present in large amount in cuttle-fish testes, were obtained as such by the same chromatographic procedure. The degree of acetylation was checked by polyacrylamide gel electrophoresis at pH 2.7 in 2.5 M urea (Panyim and Chalkley, 1969) using a 17% acrylamide concentration. The amino acid sequence of cuttle-fish histone H4 is similar to that of calf thymus H4 and three residues of lysine at positions 5, 12, and 16 were found to be acetylated (Wouters-Tyrou *et al.*, 1981).

*C.d. experiments*

C.d. measurements on core particles were performed in buffer R-0.25 M NaCl as previously described (de Murcia *et al.*, 1980). The results are presented in terms of molar ellipticity $|\theta|$ (in degrees/cm$^2$/dmol) based on the molar nucleotide concentration determined from an extinction coefficient of 6600.

*Electron microscopy*

The samples used for c.d. measurements were diluted to 0.5 $\mu$g/ml in 10 mM triethanolamine pH 7.4 and spread on carbon-coated grids positively charged, according to the method of Dubochet *et al.* (1971).

*Preparation of antisera*

Antisera against purified H2B, H2A, H3, and H4 of chicken erythrocyte were prepared by immunizing rabbits with histone-RNA complexes (3:1 w/w) as described by Stollar and Ward (1970). Antisera were also raised against the C-terminal hexapeptide of H3 (of sequence IRGERA) as described previously (Muller *et al.*, 1982). In control RIA experiments, no binding was observed between each specific histone antiserum and non-related histones used at a concentration of 400 ng histone/ml. It has been verified that histone antisera reacted about equally strongly with homologous chicken erythrocyte histones as with the corresponding calf thymus histones.

*Solid-phase RIA*

The assay was performed as described by Muller *et al.* (1982). Briefly, flexible microtitre plates were coated overnight at 37°C with 250 $\mu$l antigen in phosphate buffered saline (pH 7.4) containing 0.05% Tween 20 and 1 mM EDTA. Washed coated wells were then incubated for 3 h at 37°C with diluted antiserum. After washing, 250 $\mu$l (4 x 10$^4$ c.p.m./ml) of $^{125}$I-labelled protein A (60–100 mCi/mg) was added (Radiochemical Centre, Amersham, UK). After 1 h incubation at 37°C and extensive rinsing, the individual wells were cut out and the bound radioactivity counted in a gamma counter. The counting background was ~100 c.p.m. and was subtracted from the specific counts. The coefficient of variation of net counts observed in the same plate was ~10%.

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