Effects of histone acetylation, ubiquitination and variants on nucleosome stability

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The properties of the nucleosomes of a salt-soluble, transcriptionally active gene-enriched fraction of chicken erythrocyte chromatin were evaluated by hydroxyapatite dissociation chromatography. We have demonstrated previously that the salt-soluble, transcriptionally active gene-enriched polynucleosomes are enriched in dynamically acetylated and ubiquitinated histones, and in an atypical U-shaped nucleosome that possessed about 20% less protein than a typical nucleosome. Further, newly synthesized histones H2A and H2B exchange preferentially with the nucleosomal histones H2A and H2B of this salt-soluble chromatin fraction. Analysis of the histones eluting from the hydroxyapatite-bound chromatin demonstrated that hyperacetylated and ubiquitinated (u), including multiubiquitinated, H2A–H2B.1 dimers dissociated at lower concentrations of NaCl than unmodified dimers or dimers with histone variants H2A.Z and/or H2B.2. Cross-linking studies revealed that at least 50% of uH2B.1 was paired with uH2A. uH2A–uH2B.1 dimers dissociated at lower NaCl concentrations than H2A–uH2B.1 dimers. Hyperacetylated histone $(H3–H4)_2$ tetramers also eluted at lower concentrations of NaCl than unmodified tetramers. Our results support the idea that acetylation and ubiquitination of histones H2A and H2B.1 increase the lability of H2A–H2B.1 dimers in transcriptionally active nucleosomes. In contrast, our observations suggest that histone variants H2A.Z and H2B.2. stabilize the association of the H2A–H2B dimer in nucleosomes. The elevated lability of the H2A–H2B dimer may facilitate processes such as the exchange of these dimers with newly synthesized histones, the elongation process of transcription and transcription factor binding.

INTRODUCTION

The nucleosome, the basic repeating structural unit of chromatin, consists of two each of the core histones (H2A, H2B, H3 and H4) around which is wrapped 146 bp of DNA. The histone octamer is organized as a histone $(H3–H4)_2$ tetramer flanked by two H2A–H2B dimers (van Holde, 1988). Both transcriptionally active and repressed DNA sequences are associated with nucleosomes. However, the structures of transcriptionally active and repressed chromatin are different, with transcriptionally active nucleosomes having an atypical structure (Chen et al., 1990; Boffa et al., 1990; Locklear et al., 1990). Current evidence indicates that highly acetylated core histones, ubiquitinated H2B (uH2B), the variant H2A.Z and labile H2A–H2B dimers are characteristic features of transcriptionally active nucleosomes (Grunstein, 1990; Elgin, 1990; van Holde et al., 1992; van Daal and Elgin, 1992). It has been proposed that these histone modifications and variants alter nucleosome structure.

Dynamically acetylated histones, which attain high acetylation levels and are rapidly deacetylated, are associated principally with transcriptionally active chromatin (Ip et al., 1988; Boffa et al., 1990; Hendzel et al., 1991). It has been demonstrated that the susceptibility of nucleosomal DNA site 60 to DNAase I is increased in nucleosomes with hyperacetylated histones (Simpson, 1978; Ausio and van Holde, 1986), and hyperacetylated histones H3 and H4 reduce the linking number per nucleosome (Norton et al., 1990). These observations are consistent with the idea that the primary effect of histone H3 and H4 hyperacetylation is to weaken histone–DNA interactions in the inner 80–100 bp of the nucleosomal DNA (Ausio and van Holde, 1986). uH2B and the histone H2A variant H2A.Z also appear to be components of transcriptionally active chromatin (Ridsdale and Davie, 1987; White et al., 1988; Davie and Murphy, 1990; Davie et al., 1991). The histone H2A.Z-like variant hv1 is found in the transcriptionally active *Tetrahymena* macronucleus but not in the inactive micronucleus (White et al., 1988). Deletion of the *Drosophila* gene coding for the histone variant H2AvD, which is similar to mammalian and avian H2A.Z, is lethal (van Daal et al., 1988; van Daal and Elgin, 1992). It has been proposed that histone acetylation and/or histone U2B ubiquitination may contribute to the lability of the H2A–H2B dimer, which in turn may facilitate processes such as transcriptional elongation and transcription factor binding (Loidl, 1988; Gonzalez and Palacian, 1989; van Holde et al., 1992; Morse, 1992; Adams and Workman, 1993).

To study the effect of histone modifications and histone variants on histone–DNA interactions in nucleosomes, we used hydroxyapatite dissociation chromatography of transcriptionally active gene-enriched, 0.15 M NaCl-soluble, polynucleosomes and of bulk chromatin fragments of chicken mature and immature adult erythrocytes. Hydroxyapatite column chromatography offers a direct approach for the analysis of histone–DNA interactions as the DNA is immobilized by the resin while the histones can be eluted by a gradient of NaCl (Bloom and Anderson, 1978; Simon and Felsenfeld, 1979). Of the nucleosomal histones, the histone H2A–H2B dimer dissociates first at approx. 0.9 M NaCl, followed by the histone $(H3–H4)_2$ tetramer which dissociates between 1.2 and 2.0 M NaCl. At these salt concentrations, the trypsin-sensitive basic tails of the nucleosomal histones, which are the sites of acetylation, do not interact with the DNA (Walker, 1984; Stefanovsky et al., 1989). As nucleosome–hydroxyapatite interactions do not cause dissociation of the histones from the DNA, the method provides a means to compare the stability of nucleosomes with different modified histone forms and variants (Hirose, 1988). *In vivo* studies indicate that the H2A–H2B dimer dissociates before the

Abbreviations used: AUT, acetic acid/6.7 M urea/0.375% (w/v) Triton X-100; DTT, dithiothreitol; u, ubiquitinated; u_2, diubiquitinated.

$(H3–H4)_2$ tetramer, and this order of dissociation is reversed during nucleosome assembly (Svaren and Chalkley, 1990; Hendzel and Davie, 1990; Morse, 1992; van Holde et al., 1992). Thus the dissociation profiles of histones from hydroxyapatite-bound chromatin fragments may mimic the disassembly of nucleosomes *in situ*. We demonstrate that acetylation and ubiquitination of histones H2A and H2B.1 weaken their interaction with the $(H3–H4)_2$ tetramer and/or nucleosomal DNA, while histones H2A.Z and H2B.2 strengthen these interactions.

MATERIALS AND METHODS

Isolation and incubation of chicken erythrocytes

Mature and immature erythrocytes were isolated from adult White Leghorn chickens as described by Delcuve and Davie (1989). Dynamically acetylated histones were labelled as described by Hendzel et al. (1991).

Nuclei isolation, digestion and chromatin fractionation

Nuclei were digested and the chromatin was fractionated as described previously (Delcuve and Davie, 1989; Hendzel et al., 1991), except that incubations with micrococcal nuclease were for 20 min. Under these conditions 2.0–2.4% of the DNA was rendered acid-soluble. Briefly, the fractionation protocol included the following steps. Digested nuclei were resuspended into 10 mM EDTA/1 mM phenylmethanesulphonyl fluoride, and solubilized chromatin fragments were obtained in fraction S_E. Chromatin fraction S_E was made 150 mM in NaCl, and salt-soluble (S_{150}) and salt-insoluble (P_{150}) chromatin fractions were collected. Chromatin fragments in fraction S_{150} were size-resolved on a Bio-Gel A-5m column, obtaining a salt-soluble polynucleosome fraction (F_I and F_{II}; Ridsdale and Davie, 1987).

Hydroxyapatite chromatography

Total chromatin fragments (fraction S_E) and salt-soluble polynucleosomes were applied to a hydroxyapatite (Bio-Rad HTP) column at a ratio of 1 mg of DNA to 0.25 g of hydroxyapatite. The column was washed with 0.63 M NaCl in 0.1 M potassium phosphate, pH 6.7, removing histones H1 and H5 as well as non-histone chromosomal proteins. A linear NaCl gradient from 0.63 to 2 M NaCl in 0.1 M potassium phosphate, pH 6.7/1 mM dithiothreitol (DTT) was run through the column at a flow rate of 10 ml/h, and the conductivity of the fractions was measured. For some chromatographic runs (e.g. Figure 2), the Pharmacia f.p.l.c. system was used with a flow rate of 2 ml/min. To 1 ml of a sample of every fourth fraction was added 100 μg of 20 mg/ml casamino acid and then trichloroacetic acid was added to 20% (w/v). The precipitated proteins were collected by centrifugation. The precipitate was twice resuspended in acetone and then lyophilized. The sample was dissolved in 25 μl of AUT sample buffer with 50% (v/v) 2-mercaptoethanol (Nickel et al., 1987), and 20 μl was loaded on to the AUT [acetic acid/6.7 M urea/0.375% (w/v) Triton X-100]/polyacrylamide gel. Alternatively, pooled fractions were dialysed against deionized distilled water and lyophilized. The samples were redissolved in 1 mM DTT.

Analysis of protein samples

Histones were electrophoretically resolved on one-dimensional SDS or AUT, or two-dimensional (AUT into SDS), 15%-polyacrylamide gels (Nickel et al., 1987). Chicken erythrocyte histone variants and modified forms described by Urban et al. (1979) were identified on an acetic acid/0.375% (w/v) Triton X-100/15% polyacrylamide gel containing a transverse urea gradient (0–8 M) (Strickland et al., 1981). Immunochemical staining of ubiquitin–histone conjugates was performed as described previously (Nickel et al., 1987; Delcuve and Davie, 1992). For detection of the anti-ubiquitin antibodies, either alkaline phosphatase-conjugated goat anti-rabbit antibody or horseradish peroxidase-conjugated goat anti-rabbit antibody (Amersham ECL Western Blotting system) were used. Fluorography was performed as described by Hendzel and Davie (1989).

Cross-linking of H2A–H2B dimers

Pooled column fraction I (H2A–H2B dimers) from hydroxyapatite-bound salt-soluble polynucleosomes (see Figure 1) was dialysed against 10 mM sodium borate, pH 10/1 M NaCl. The sample was concentrated to 0.27 A_{230} unit/ml. Dimethyl 3,3′-dithiobispropionimidate was added to 1 mg/ml and the mixture was incubated at room temperature for 30 min. Trichloroacetic acid was added to 20% (w/v), and the precipitate was collected by centrifugation. The precipitate was resuspended first in acetone/0.1 M HCl, then in acetone, and lyophilized. The cross-linked and non-cross-linked control samples were electrophoresed on AUT/10%-polyacrylamide gels. The sample lanes of 8-anilino-1-naphthalenesulphonic acid-stained gels that were visualized under u.v. light (Davie, 1982) were excised and treated with 10 ml of 10 mM (3-[cyclohexylamino]-1-propanesulphonic acid), pH 10/5% (v/v) 2-mercaptoethanol for 30 min. The histones of the gel strips were electrophoresed on a second-dimension AUT/15%-polyacrylamide gel. To cross-link histones in polynucleosomes the following procedure was used. Dry CM-Sephadex (30 mg/ml) was added to the polynucleosomes, which were in 10 mM Tris/HCl, pH 8.0/1 mM EDTA/150 mM NaCl, followed by the addition of NaCl to 0.35 M from a 4 M stock. This procedure removes the H1 and H5 histones (Ridsdale et al., 1990). The stripped polynucleosomes were dialysed against 100 mM triethanolamine, pH 8.4. Polynucleosomes (1 ml; 10 A_{260} units/ml) were treated with dimethyl 3,3′-dithiobispropionimidate (4 mg/ml) for 2 h at 0 °C. An equivalent volume of 2 × AUT sample buffer containing 20 mg/ml protamine (grade X from salmon; Sigma) was then added, and the protamine–DNA precipitate was removed by centrifugation. The supernatant was dialysed against water at 4 °C, lyophilized and dissolved in 200 μl of water. To 50 μl of sample was added 20 μl of 100% glycerol, and 60 μl was applied to the AUT/10%-polyacrylamide gel.

RESULTS

Hydroxyapatite column chromatography of total and salt-soluble chromatin fragments

Chicken mature erythrocyte nuclei were incubated with micrococcal nuclease, and the fragmented chromatin was fractionated by a low-ionic-strength procedure. Chromatin fraction S_E contained the bulk of the chromatin fragments (approx. 90% of the nuclear DNA), while the transcriptionally active gene-enriched, 0.15 M NaCl-soluble polynucleosomes represented approx. 1% of the nuclear DNA (Delcuve and Davie, 1989). The 0.15 M NaCl-soluble polynucleosomes are enriched in transcriptionally active DNA sequences (e.g. β-globin and histone H5), dynamically acetylated core histones, ubiquitinated histone H2B, multi-ubiquitinated H2A and histone H2A.Z (Ridsdale and Davie, 1987; Delcuve and Davie, 1989; Hendzel et al., 1991). Total

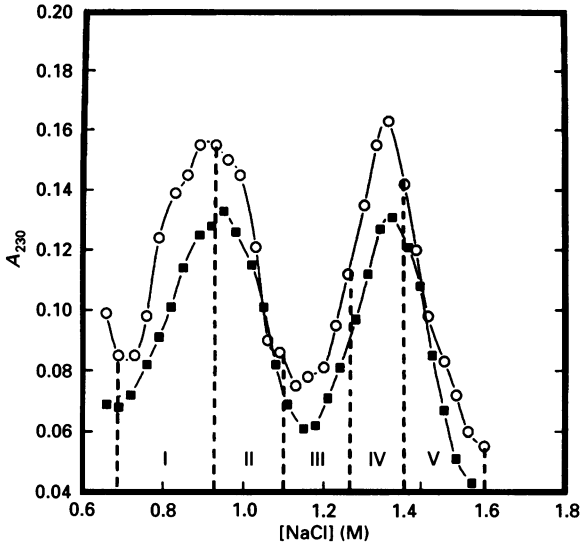

Figure 1 Hydroxyapatite dissociation chromatography of total and salt-soluble polynucleosomes

Total chromatin fragments (■; fraction S_E; 60 A_{260} units) or salt-soluble polynucleosomes (○; fractions F_I and F_{II}; 75 A_{260} units) were applied to a hydroxyapatite column. A linear gradient from 0.63 M to 2 M NaCl in 0.1 M potassium phosphate (pH 6.7)/1 mM DTT was run through the column. The conductivity of each fraction was measured and the equivalent NaCl concentration determined. The fractions that were pooled are shown (I–V).

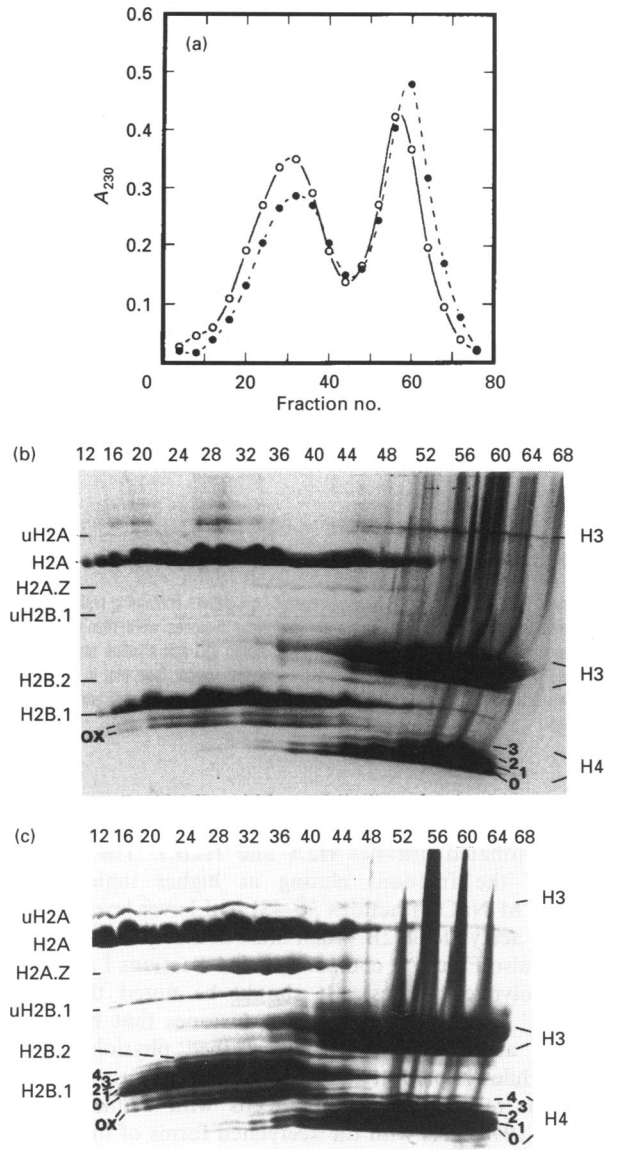

Figure 2 Effects of histone modifications and histone variants on nucleosome properties

(**a**) Chromatographic profiles of histones eluting from hydroxyapatite-bound total chromatin fragments (●; 250 A_{260} units) and salt-soluble polynucleosomes (○; 250 A_{260} units). A linear gradient from 0.63 M to 2 M NaCl in 0.1 M potassium phosphate (pH 6.7)/1 mM DTT was run through the column, and 2.5 ml fractions were collected. The histones in every fourth fraction (1 ml of each fraction) were precipitated with trichloroacetic acid as described in the Materials and methods section. The histones were electrophoretically resolved on AUT/15%-polyacrylamide gels (**b**, total chromatin; **c**, salt-soluble polynucleosomes). The gels were stained with Coomassie Blue. 0, 1, 2, 3 and 4 correspond to the un-, mono-, di-, tri- and tetra-acetylated species of histones H2B.1 and H4. The oxidized forms of histone H2B.1 are marked as ox.

chromatin fragments and salt-soluble polynucleosomes were applied to a hydroxyapatite column, and the histones were dissociated from the DNA with increasing concentrations of NaCl. Figure 1 shows representative elution profiles of the two groups of chromatin fragments. Two peaks of A_{230}-absorbing material were observed for both profiles. The first peak of A_{230}-absorbing material (histones H2A and H2B; see Figure 2) eluted at 0.91 ± 0.01 M NaCl ($n = 6$) from total chromatin and at 0.93 ± 0.02 M NaCl ($n = 6$) from the salt-soluble polynucleosomes, while the second peak (histones H3 and H4; see Figure 2) eluted at 1.42 ± 0.02 M NaCl ($n = 6$) from total chromatin and at 1.38 ± 0.03 M NaCl ($n = 6$) from the salt-soluble polynucleosomes. At these salt concentrations, the core histones would dissociate as H2A–H2B dimers and $(H3–H4)_2$ tetramers (Eickbush and Moudrianakis, 1978). Thus the histone H2A–H2B dimer and histone $(H3–H4)_2$ tetramer dissociated from the total chromatin fragments and the salt-soluble polynucleosomes at similar salt concentrations. However, a significant proportion of the histone H2A–H2B dimers dissociated from the salt-soluble polynucleosomes at lower salt concentrations than those from total chromatin (see also Figure 2). Of the A_{230}-absorbing material dissociating from the hydroxyapatite-bound chromatin fragments between 0.65 and 1.12 M NaCl, 60% and 53% of this material was released from salt-soluble polynucleosomes and total chromatin fragments respectively, between 0.65 and 0.93 M NaCl. This was a representative result of 10 chromatographic profiles of histones eluting from hydroxyapatite-bound total or salt-soluble polynucleosomes.

Hatch et al. (1983) demonstrated that the H2A and uH2B histones and the minor histone variant H2A.Z were present in native H2A–H2B dimers. To determine the salt concentration required to dissociate H2A–H2B dimers with histone variants or modified forms from hydroxyapatite-bound chromatin fragments, proteins of individual column fractions were electrophoretically resolved on AUT/polyacrylamide gels. Figure 2 shows that histones H2A and H2B eluted first, followed by histones H3 and H4. The identity of the histone forms was determined by their electrophoretic migration on AUT/15%-polyacrylamide gels containing a transverse urea gradient (0–8 M) and on two-dimensional 15%-polyacrylamide gels (AUT into SDS). The H2A–H2B dimers that dissociated between 0.70 and 0.93 M NaCl (fractions 12–32) from the hydroxyapatite-

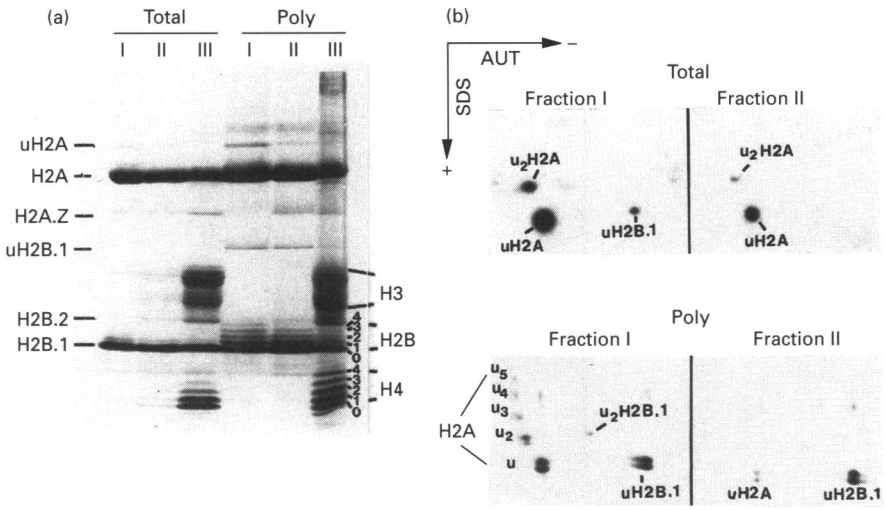

Figure 3 Effects of ubiquitination on nucleosome properties

(**a**) Coomassie Blue-stained AUT/15%-polyacrylamide gel pattern containing histones (17 μg) that dissociated from the hydroxyapatite-bound bulk chromatin (Total) and salt-soluble polynucleosomes (Poly) (fractions I, II and III; see Figure 1). (**b**) These histones were resolved by two-dimensional gel electrophoresis (AUT into SDS), electrophoretically transferred to nitrocellulose, and immunochemically stained for ubiquitin with anti-ubiquitin IgG and alkaline phosphatase-conjugated goat anti-rabbit antibody. The fraction analysed is indicated. The colour development time to visualize the ubiquitinated histones of total chromatin was longer than that to detect ubiquitinated histones of salt-soluble polynucleosomes. 0, 1, 2, 3 and 4 correspond to the un-, mono-, di-, tri-, and tetra-acetylated species of histones H2B and H4. The multiubiquitinated histone species are labelled u_2, u_3, u_4, and u_5, representing the attachment of two, three, four and five ubiquitins respectively.

bound salt-soluble polynucleosomes contained highly acetylated and ubiquitinated histones H2A and H2B.1. The H2A–H2B dimers of the fractions eluting at higher ionic strengths (0.93–1.23 M NaCl; fractions 36–48) had lower levels of uH2A and highly acetylated H2B.1 than those in the fractions preceding them (see also Figure 3, compare pooled fractions I and II, salt-soluble polynucleosomes). [It should be noted that 'highly acetylated' histones refers to those histones that have a high degree of acetylation under unperturbed physiological conditions, while 'hyperacetylated' histones refers to the level of acetylation reached by treating cells with sodium butyrate (Csordas, 1990).] As with the acetylated forms of histone H2B, the histone H4 eluting first from the hydroxyapatite-bound salt-soluble polynucleosomes had greater levels of the highly acetylated forms than the histone H4 eluting at higher salt concentrations (compare the levels of tetra-acetylated histone H4 in fractions 48–56 with those in fractions 64 and 68).

Histone H2A–H2B dimers that contained histone variants H2A.Z or H2B.2 did not dissociate from the total chromatin fragments or salt-soluble polynucleosomes until the salt concentration reached 0.9–1.0 M NaCl (Figures 2 and 3). Fraction 32 had the highest concentration of H2A–H2B dimers (both total and salt-soluble polynucleosomes; Figure 2), while fraction 44 from total chromatin fragments and fractions 36–40 from salt-soluble polynucleosomes had the greatest amount of H2A.Z (Figure 2). For histone H2B.2, fractions 48 and 40 from the total and salt-soluble polynucleosomes respectively had the highest amounts of this histone variant.

It was possible that the H2A–H2B dimers containing histone variants were eluting at higher salt concentrations than the bulk of the H2A–H2B dimers by binding to the column matrix following their dissociation from chromatin. Thus histones isolated from salt-soluble polynucleosomes were applied to the hydroxyapatite column which was equilibrated with 0.63 M NaCl/0.1 M potassium phosphate, pH 6.7. None of the modified histone forms or histone variants bound to the column. In agreement with the results of Bloom and Anderson (1978) and Hirose (1988), our observations show that the hydroxyapatite matrix is not involved in the fractionation of the histone variants or modified histone forms.

Elution of multiubiquitinated histones from hydroxyapatite-bound chromatin fragments

To analyse further the dissociation of the ubiquitinated histones from the hydroxyapatite-bound chromatin fragments, the histones in the column fractions were resolved by two-dimensional gel electrophoresis (AUT into SDS) and transferred to nitrocellulose, and the ubiquitinated histone forms were detected by immunochemical staining with an anti-ubiquitin antibody. The histones of pooled fraction I (total or salt-soluble polynucleosomes; see Figure 1) were enriched in uH2A and uH2B.1 when compared with those of pooled column fractions II and III (Figure 3). The differences in the content of uH2A and uH2B.1 between pooled fractions I and II of salt-soluble polynucleosomes were more striking for uH2A than with uH2B.1. This differential elution of the ubiquitinated histones was also evident in the AUT gel pattern shown in Figure 2 (salt-soluble polynucleosomes; compare fractions 32 and 36).

Histones H2A and H2B.1 can be multiubiquitinated. In multiubiquitinated histone H2A, ubiquitin is arranged as a chain of ubiquitin molecules joined to each other by isopeptide bonds to a ubiquitin molecule that is attached to the ϵ-amino group of lysine-119 of histone H2A (Nickel and Davie, 1989). Multiubiquitinated histone H2A with up to five attached ubiquitins and diubiquitinated histone H2B.1 (u_2H2B.1) were detected in the histones that dissociated from the salt-soluble polynucleosomes into pooled column fraction I (Figure 3). Histones that dissociated from these chromatin fragments at higher ionic strengths (pooled column fractions II and III) had substantially lower levels of the multiubiquitinated histone forms. As with the salt-soluble polynucleosomes, pooled fraction I of total chroma-

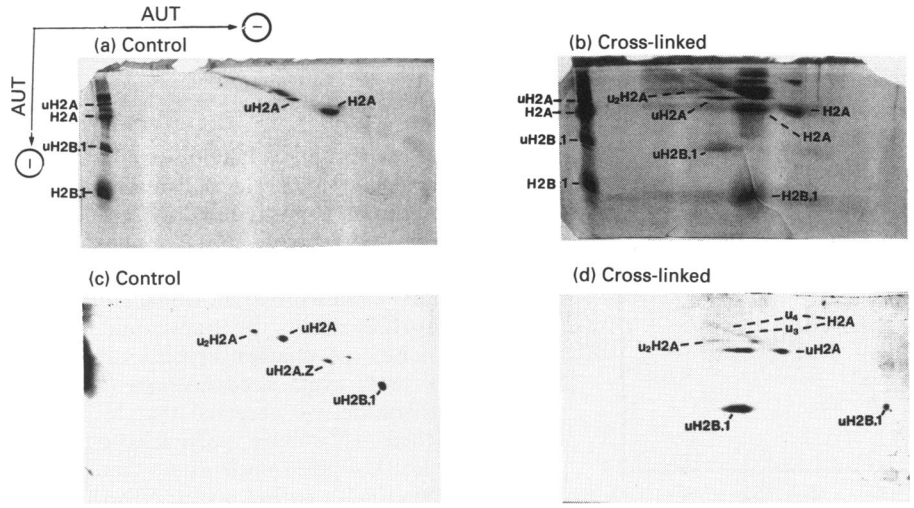

Figure 4 Identification of dimers containing uH2A and uH2B.1

Panels (**a**) and (**b**) show silver-stained two-dimensional gel patterns (AUT into AUT) containing control (40 μg) and cross-linked (60 μg) H2A–H2B.1 dimers that dissociated from the hydroxyapatite-bound salt-soluble polynucleosomes (fraction I; see Figure 1). Panels (**c**) and (**d**) show two-dimensional gel patterns (control and cross-linked H2A–H2B.1 dimers respectively) that were electrophoretically transferred to nitrocellulose and immunochemically stained for ubiquitin with anti-ubiquitin IgG and horseradish peroxidase-conjugated goat anti-rabbit antibody (ECL Western Blotting system). The multiubiquitinated histone species are labelled u_2, u_3 and u_4, representing the attachment of two, three and four ubiquitins respectively.

tin had a higher concentration of u_2H2A than did fraction II. This was a representative result of four experiments.

Cross-linking of H2A–H2B dimers from salt-soluble polynucleosomes

The H2A and H2B histones eluting in fractions 12–32 from the hydroxyapatite-bound salt-soluble polynucleosomes contained both uH2A and uH2B.1 (Figure 2; see also pooled fraction I in Figure 3). To investigate whether uH2A and uH2B.1 were paired together in a H2A–H2B dimer, pooled column fraction I H2A–H2B.1 dimers were cross-linked with dimethyl 3,3′-dithiobispropionimidate, a cleavable bifunctional cross-linker, and electrophoretically resolved on an AUT/10%-polyacrylamide gel. The sample lane was treated with a reducing agent before being electrophoresed on a second-dimension AUT/15%-polyacrylamide gel. Figure 4(a) shows the two-dimensional pattern of the control (not cross-linked) sample. In the second-dimension AUT gel pattern, the proteins were positioned on a diagonal. Figure 4(b) shows the silver-stained gel pattern containing the cross-linked H2A–H2B.1 dimers. Histone H2A was cross-linked to H2B.1. This result is consistent with the H2A and H2B histones eluting from the hydroxyapatite-bound salt-soluble polynucleosomes as dimers. In addition, the two-dimensional pattern shown in Figure 4(b) also indicates that uH2B.1 was cross-linked to uH2A. In a second set of control experiments, the dimers were cross-linked and then treated with reducing agent prior to being electrophoresed on the first-dimension gel. The majority of the histones migrated on the second-dimension diagonal as shown in Figure 4(a). However, several bands migrated off the diagonal in a position above H2A, similar to those shown in Figure 4(b). Bands migrating below the diagonal in the second-dimension gel pattern containing the control sample were not observed (results not shown). The two-dimensional gel patterns containing cross-linked and control H2A–H2B.1 dimers were analysed in Western blotting experiments with the anti-ubiquitin antibody. Figure 4(c) shows that u_2H2A, uH2A, uH2A.Z and uH2B.1 were positioned on a diagonal. Figure 4(d) shows the positions of the ubiquitinated histones that were not cross-linked and migrated on the diagonal, and of those that were cross-linked to other proteins and migrated away from the diagonal. The two-dimensional gel pattern of Figure 4(d) shows that some of the uH2A, uH2B.1 and uH2A.Z (seen on the autoradiograph but not the photograph) was not cross-linked to other proteins. The patterns also demonstrated that the majority of uH2B.1 was cross-linked to uH2A. u_2H2A did not appear to be cross-linked to uH2B.1. Considering that pooled column fraction I contained approximately one-half (at a minimum) of the total uH2B.1 in salt-soluble polynucleosomes (Figure 3), at least 50% of the uH2B.1 in these transcriptionally active gene-enriched chromatin fragments would be paired with uH2A. This was a representative result of four experiments. These cross-linking experiments were also done with salt-soluble polynucleosomes (see the Materials and methods section). Although the efficiency of cross-linking was considerably less than that of isolated H2A–H2B dimers, cross-linking of uH2A to uH2B.1 was observed (results not shown).

Elution of dynamically acetylated histones from hydroxyapatite-bound chromatin fragments

In chicken immature adult erythrocytes, approx. 4% of the modifiable histone lysine sites participate in dynamic acetylation (Zhang and Nelson, 1986). We and others have demonstrated that dynamically acetylated nucleosomal histones are complexed primarily to transcriptionally active DNA (Ip et al., 1988; Boffa et al., 1990; Hendzel et al., 1991). Thus, monitoring the dissociation of labelled hyperacetylated histones from hydroxyapatite-bound chromatin fragments provides an indirect method of monitoring the dissociation of histones from transcriptionally active nucleosomes.

To label the dynamically acetylated histones, chicken immature erythrocytes were pulse-labelled with [^{3}H]acetate for 15 min followed by a 60 min chase in Swim's S-77 medium containing 10 mM sodium butyrate, an inhibitor of histone deacetylase. Figure 5 shows the elution of the labelled hyperacetylated histones

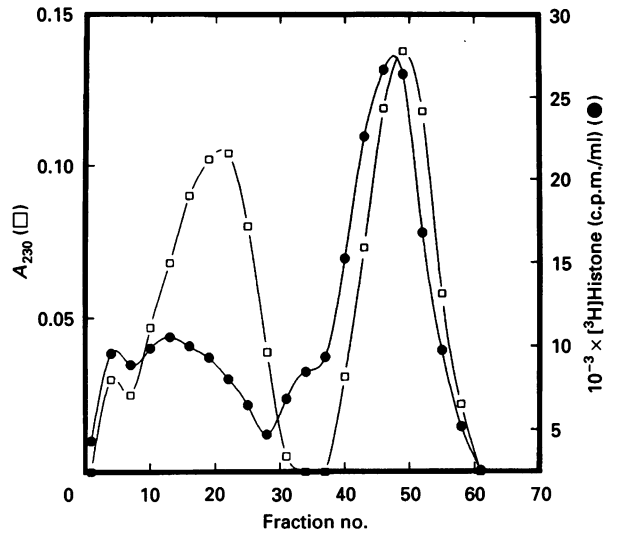

Figure 5 Effects of histone hyperacetylation on nucleosome properties

Dynamically hyperacetylated histones were labelled with [^{3}H]acetate as described in the Materials and methods section. The labelled bulk chromatin fragments (80 A_{260} units) were applied to a hydroxyapatite column. A linear 200 ml gradient from 0.63 M to 2 M NaCl in 0.1 M potassium phosphate (pH 6.7)/1 mM DTT was run through the column. Fractions of 2 ml were collected.

relative to histones of total chromatin (fraction S_E) fragments, and Figure 6 displays the labelled histone species electrophoretically resolved on AUT/polyacrylamide gels. Labelled hyperacetylated histones H2A and H2B.1 dissociated from the chromatin fragments at lower salt concentrations than the majority of histones H2A and H2B.1. Furthermore, the salt concentration required to separate the dynamically acetylated histones H3 and H4 from the DNA was lower than that required to extract the bulk of these histones. Of the four core histones, the acetylated forms of histone H4 were the best resolved (Figure 6). The percentage of labelled histone H4 that was tetra-acetylated was determined by densitometric scanning of the fluorogram. Approx. 40, 30 and 18% of the labelled acetylated histone H4 species were tetra-acetylated in the histones of pooled column fractions III, IV and V respectively. This was a representative result of two experiments.

DISCUSSION

It has been proposed that loss of a histone H2A–H2B dimer is an intermediate step in the elongation process of transcription and transcription factor-induced nucleosome disassembly (Baer and Rhodes, 1983; Loidl, 1988; Gonzalez and Palacian, 1989; Morse, 1992; van Holde et al., 1992; Adams and Workman, 1993). In support of this proposal, we demonstrated that *in situ* histone H2A–H2B dimers of transcriptionally active gene-enriched chromatin of chicken erythrocytes preferentially exchanged with newly synthesized histones (Hendzel and Davie, 1990). Furthermore, we observed that transcriptionally active gene-enriched, salt-soluble polynucleosomes were enriched in an atypical U-shaped nucleosome that possessed about 20% less protein than a typical nucleosome (Locklear et al., 1990). This reduction in protein mass would be consistent with the loss of a histone H2A–H2B dimer. Baer and Rhodes (1983) reported that RNA polymerase II bound selectively to nucleosomes that were enriched in transcriptionally active DNA sequences and missed an H2A–H2B dimer. Interestingly, these nucleosomes were depleted in uH2A. Using hydroxyapatite dissociation chromatography to analyse histone–DNA contacts in transcriptionally active gene-enriched nucleosomes, we provide evidence that acetylation and ubiquitination of histones H2A and H2B.1 weaken the binding of the histone H2A–H2B dimer in transcriptionally active gene nucleosomes.

Highly acetylated histone H2B.1 as well as labelled hyperacetylated histones H2A and H2B.1 (presumably from transcriptionally active chromatin) eluted from the hydroxyapatite-bound, salt-soluble polynucleosomes at lower NaCl concentrations than did the bulk of the unacetylated histone H2B.1. Several investigators have shown that the thermal denaturation profiles of control and hyperacetylated nucleosomes are different (Bode et al., 1980; Ausio and van Holde, 1986). The first transition of the biphasic melting profile occurs at a lower temperature for hyperacetylated nucleosomes than for control nucleosomes. The first transition is thought to be the melting of DNA 25 to 30 bp from the end of the nucleosomal DNA. Ausio et al. (1989) have presented evidence that indicates that the tails of histones H2A and H2B interact with a 10 bp region near the

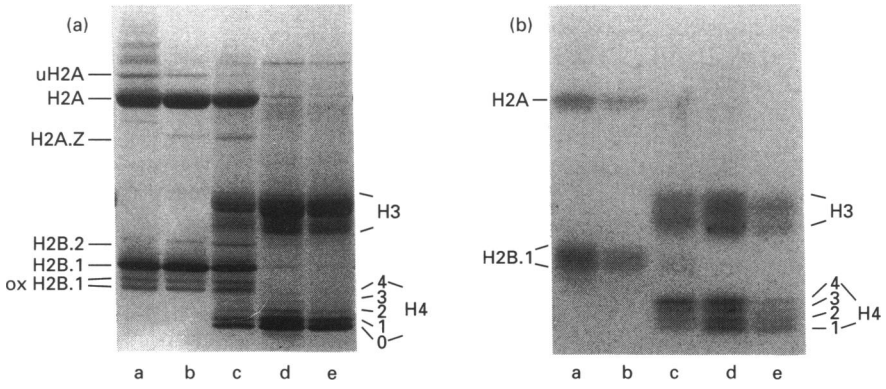

Figure 6 Dissociation of hyperacetylated histones from hydroxyapatite-bound chromatin fragments

(**a**) Coomassie Blue stained gel pattern containing histones (6.5 μg) from the column fractions shown in Figure 5 (lanes a, b, c, d, and e contain histones from fractions 3–15, 16–27, 28–40, 41–50 and 51–57 respectively). (**b**) Accompanying fluorogram. 0, 1, 2, 3, and 4 correspond to the un-, mono-, di-, tri-, and tetra-acetylated species of histones H2B and H4 respectively. The oxidized forms of histone H2B.1 are marked as ox.

ends of the nucleosomal DNA. These observations suggest that acetylation of histones H2A and H2B.1 weakens the histone–DNA interactions occurring between the histone-H2A–H2B.1 dimer and the ends of the nucleosomal DNA, facilitating the dissociation of a dimer.

Our results show that the first H2A–H2B dimers to dissociate from total chromatin fragments and transcriptionally active gene-enriched polynucleosomes at salt concentrations between 0.65 and 0.93 M NaCl contained dimers of uH2A–uH2B.1. In uH2A and uH2B, the C-terminal glycine of ubiquitin is attached to the ε-amino group of lysine-119 (H2A) or lysine-120 (H2B) in the C-terminal portion of the molecule. Removal of the C-terminus of H2A or H2B reduces the affinity of the H2A–H2B dimer for the $(H3–H4)_2$ tetramer (Burlingame et al., 1985; Eickbush et al., 1988). Thus the attachment of ubiquitin to the C-terminal portion of H2A and H2B may destabilize the interaction between the H2A–H2B dimer and $(H3–H4)_2$ tetramer. Furthermore, multiubiquitination of H2A and H2B may also affect the interaction between the dimer and the tetramer. The observation that multiubiquitinated histones H2A and H2B.1 dissociated first from the hydroxyapatite-bound, salt-soluble polynucleosomes supports this idea. Thus acetylation and ubiquitination of histones H2A and H2B.1 may promote disruption of histone–DNA and histone (H2A–H2B.1 dimer)–[$(H3–H4)_2$ tetramer] interactions. Further, this idea provides an explanation for the absence of uH2A in nucleosomes that were bound to RNA polymerase II and had lost an H2A–H2B dimer (Baer and Rhodes, 1983).

Recently, Moehs et al. (1992) provided evidence that wheat histones H2A and H2B enhanced the stability of histone octamers. It was proposed that the extended C-termini of wheat H2A histones may have accounted for the increased stability of the plant octamer. Their results indicated that histone H2A and H2B variants may affect the lability of histone H2A–H2B dimers in nucleosomes. Our results provide support for this idea. We found that the interaction of chicken histone H2A.Z and histone H2B.2 with the $(H3–H4)_2$ tetramer and/or nucleosomal DNA was stronger than that of histones H2A and H2B.1. The amino acid sequence of histone H2B.2 is very similar to that of histone H2B.1, differing at positions 31, 32 and 60 (Grandy and Dodgson, 1987). The H2A.Z and H2A amino acid sequences differ markedly in two regions, at H2A.Z positions 73–82 and 97–127 (Harvey et al., 1983; Hatch and Bonner, 1988). Thus histone modifications and histone variants (H2A.Z and H2B.2) may generate asymmetry in nucleosomes with respect to the binding of the H2A–H2B dimers. For example, a histone H2A–H2B dimer consisting of a uH2A–uH2B.1 dimer would dissociate more readily than a dimer of H2A.Z and H2B.2, aiding in the generation of a nucleosome with a histone hexamer devoid of ubiquitinated histones.

In agreement with the observations of Hirose (1988), our results demonstrate that acetylation of histones H3 and H4 weakens the interaction between the $(H3–H4)_2$ tetramer and nucleosomal DNA under high salt concentrations. The stability of transcriptionally active gene nucleosomes could be monitored indirectly by following the dissociation of labelled, dynamically acetylated histones. We observed that labelled hyperacetylated histones H3 and H4 eluted at lower NaCl concentrations than the bulk of these unmodified histones. Furthermore, we demonstrated that highly acetylated histone H4 dissociated from hydroxyapatite-bound total chromatin fragments and transcriptionally active gene-enriched, salt-soluble polynucleosomes at lower concentrations of NaCl than the majority of the unacetylated histone H4. These observations are consistent with the idea that high acetylation levels of histones H3 and H4 weaken histone–histone and histone–DNA contacts in transcriptionally active gene nucleosomes (Ausio and van Holde, 1986; Hirose, 1988).

In summary, our results provide evidence that acetylation and ubiquitination of histones H2A and H2B.1 contributes to the lability of H2A–H2B dimers in transcriptionally active nucleosomes, facilitating the initial steps of nucleosome disassembly during transcriptional elongation or transcription factor binding. Acetylation of the histone $(H3–H4)_2$ tetramer would further weaken histone–DNA contacts, aiding in the displacement of the $(H3–H4)_2$ tetramer and completing the removal of the nucleosome. Alternatively, histone acetylation and ubiquitination may sufficiently alter histone–DNA contacts and modify nucleosome structure, allowing the binding of transcription factors to atypical nucleosomes (Lee et al., 1993).

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