Reconstitution of native-like nucleosome core particles from reversed-phase-HPLC-fractionated histones

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We have reconstituted nucleosome core particles from reversed-phase-HPLC-purified chicken erythrocyte core histones and 145 bp random-sequence DNA fragments. Characterization of the resulting nucleoprotein complexes by sedimentation velocity, CD and DNase I footprinting showed that they are structurally indistinguishable from native nucleosome core particles. Furthermore, we have shown that the ability to reproduce these native-like structural features in these reconstituted nucleosome core particles is basically independent of the biological source or the method used (i.e. salt versus acid) for the extraction of histones before their HPLC fractionation. The usefulness and relevance of this approach for the reconstitution of native-like chromatin structures from histone types (histone variants/post-translationally modified histones), which are usually available only in relatively small amounts, is discussed.

INTRODUCTION

Reversed-phase HPLC (RP-HPLC) has become one of the most employed chromatographic techniques for protein fractionation. The high resolution and the speed with which protein and peptide separation is achieved are among the reasons for its increasing popularity. However, there is concern about the possible loss of biological activity and alteration of the native-like structural features of the proteins purified in this way [1].

In the case of histones, the efficiency of RP-HPLC chromatographic separation of each of the individual histone fractions (H1, H2A, H2B, H3 and H4) is only second to a gel-filtration liquid-chromatography fractionation that uses long columns of BioGel P-60 (Bio-Rad, Hercules, CA, U.S.A.) [2]. However, this later separation is enormously time consuming, taking several days, and requires large amounts of starting sample (15–20 mg/cm^2) [3]. In contrast, complete fractionation of histones can be quickly achieved (1–2 h) by RP-HPLC and only microgram amounts of starting sample are required. Furthermore, under optimal conditions, it is possible to separate the histone variants of some of the individual histone fractions, such as H2A.1 and H2A.2 [4]. Several methods of RP-HPLC of histones have been developed [4–13]. In most of these, trifluoroacetic acid in the range 0.1–0.3 % (v/v) is used in the mobile phase and acetonitrile (in a continuous or stepwise gradient) is used as an organic modifier. The use of high concentrations of acetonitrile, which is known to be very destructive towards the secondary and tertiary structure of proteins [14,15], raises serious concerns and has restricted the use of RP-HPLC-purified histones for chromatin reconstitution experiments. In the present paper we address this concern and show that indeed RP-HPLC-purified histones can be used in the reconstitution of native-like nucleosome core particles regardless of the biological source or the method of extraction of the histones (acid or salt) before their HPLC purification.

MATERIALS AND METHODS

Core histones and DNA

Chicken erythrocyte core histones were prepared from chicken erythrocyte nucleosome core particles as described elsewhere [16]. Histones were then extracted with salt (2 M NaCl) or with acid (0.4 M HCl).

Salt extraction

Nucleosome core particles (approx. 20 mg) in 25 mM NaCl/10 mM Tris/HCl/0.5 mM EDTA (pH 7.5)/tosyl-lysylchloromethane (10 μg/ml) were loaded onto a hydroxyapatite column (1.5 cm × 15 cm) that had been equilibrated with 0.1 M potassium phosphate (pH 6.8) at a flow rate of 10 ml/h, and the core histones were eluted with 2 M NaCl in 0.1 M potassium phosphate buffer (pH 6.8)/1 mM dithiothreitol (DTT) as described previously [17]. The 145 bp random-sequence DNA of the nucleosome core particles was eluted with 0.5 M potassium phosphate buffer (pH 6.8). The DNA fraction thus obtained was dialysed overnight at 4 °C against 10 mM Tris/HCl/1 mM EDTA (pH 7.5). After dialysis, the DNA solution was brought to 0.3 M sodium acetate and precipitated overnight with 2 vol. of ethanol at −20 °C.

Acid extraction

Nucleosome core particles in low salt (25 mM NaCl/20 mM Tris/HCl/0.5 mM EDTA, pH 7.5) were brought to 0.4 M HCl by addition of an equal volume of 0.8 M HCl and stirred for 1 h at 4 °C. The precipitated DNA was removed by centrifugation (10000 ***g*** for 10 min at 4 °C), and the supernatant (consisting of the histone extract) was precipitated with 6 vol. of acetone overnight at 4 °C or for 1 h at −20 °C.

In addition to chicken erythrocytes, alligator (*Alligator mississipiensis*) testis [18] and lamprey (*Lampetra tridentatus*) testis [19] were also used as a source of histones, which were obtained by acid extraction with 0.4 M HCl.

RP-HPLC purification of histones

Salt- or acid-extracted histones from different sources (see above) (approx. 8–10 mg) were dissolved in 1 ml of 25 % (v/v) acetonitrile/1 % (v/v) trifluoroacetic acid and were injected on to a 1 cm × 25 cm, 5 μm C_4 Vydac column and eluted at 3 ml/min using an acetonitrile gradient [20].

Abbreviations used: RP-HPLC, reversed-phase HPLC; DTT, dithiothreitol.

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Reconstitution of the RP-HPLC-fractionated histones

After RP-HPLC fractionation of the salt- or acid-extracted histones, the fractions corresponding to the core histones were pooled together and processed in different ways as discussed in the Results section.

Nucleosome core particle reconstitution

Histones from different sources and 145 bp random-sequence DNA in 2 M NaCl/10 mM Tris/HCl (pH 7.5)/0.5 mM EDTA/1 mM DTT were mixed together in stoichiometric amounts (histone/DNA, 1.13:1 w/w) and the nucleosome complexes were reconstituted at 4 °C by salt-gradient dialysis [21]. The concentrations of the core histones and DNA were determined spectrophotometrically on a Cary spectrophotometer (Varian Techtron). An absorption coefficient at 260 nm, $A_{260} = 220\ cm^2 \cdot mg^{-1}$, was used for the DNA and $A_{230} = 4.2\ cm^2 \cdot mg^{-1}$ for the core histones [22].

Nucleosome core particles reconstituted from RP-HPLC-fractionated acid-extracted histones were fractionated on sucrose gradients [23] and dialysed against 0.1 M NaCl/20 mM Tris/HCl (pH 7.5)/0.5 mM EDTA, before their analysis by sedimentation velocity and DNase I footprinting.

Analytical ultracentrifuge analysis

Sedimentation velocity analysis was carried out in a Beckman XL-A analytical ultracentrifuge as described elsewhere [16].

CD

CD spectra were recorded at 20 °C on a Jasco J-720 spectropolarimeter as described previously [23]. For the calculation of the mean residue molecular ellipticity, the M_r values of the average nucleotide and amino acid residue used were 331 and 111 [24] respectively.

DNA labelling and DNase I footprinting

Approx. 30 μg of nucleosome core particles were 5′-end labelled with [γ-^{32}P]ATP as described elsewhere [16]. Immediately thereafter the sample was dialysed using a Centricon 30 (Amicon, Beverly, MA, U.S.A.) with 10 mM NaCl/10 mM Tris/HCl (pH 7.5)/1 mM EDTA buffer, to remove the excess [γ-^{32}P]ATP. The 5′-end labelled nucleosome sample was then digested with DNase I on ice as described in [16].

PAGE

Histones were analysed on SDS/PAGE according to Laemmli [25]. The DNA fragments from DNase I digestion of γ-^{32}P-end labelled nucleosomes were analysed on 10% (w/v) acrylamide (acrylamide/bisacrylamide ratio 19:1, w/w, and 7 M urea) denaturing gels as described in [16].

RESULTS

Nucleosome core particle reconstitution upon renaturation of histones by guanidinium chloride treatment

The main aim of this work was to explore the possibility of reconstituting native-like nucleosome core particles from RP-HPLC-purified histones. In the course of this analysis we checked several of the factors that could affect the structure of histones during the reconstitution procedure. Figure 1 summarizes the main reconstitution strategies followed. Following RP-HPLC fractionation, the different core histone fractions were pooled together and freeze-dried. The freeze-dried histone powder was aliquoted into 3–4 mg fractions. One of these fractions was then dissolved in 1 ml of distilled water and then brought to 2 M NaCl/50 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 mM DTT (buffer A) by addition of an equal volume of a 4 M stock solution (fraction C in Figure 1). Alternatively a similar amount of freeze-dried powder was directly dissolved in 2 ml of 6 M guanidinium chloride/20 mM β-mercaptoethanol in 50 mM Tris/HCl (pH 7.6) buffer and incubated for 30 min at room temperature. The sample was then dialysed at 4 °C against 2 l of distilled water for 3–4 h, and then the dialysis bag was transferred to 2 l of buffer A and the dialysis continued overnight at 4 °C (fraction D in Figure 1). As a control we used the nucleosome core histones directly recovered from the hydroxyapatite column which had not been subjected to HPLC fractionation (fraction B in Figure 1). The histones from fractions B, C and D were then combined with stoichiometric amounts (see the Materials and methods section) of 145 bp DNA (recovered from the hydroxyapatite

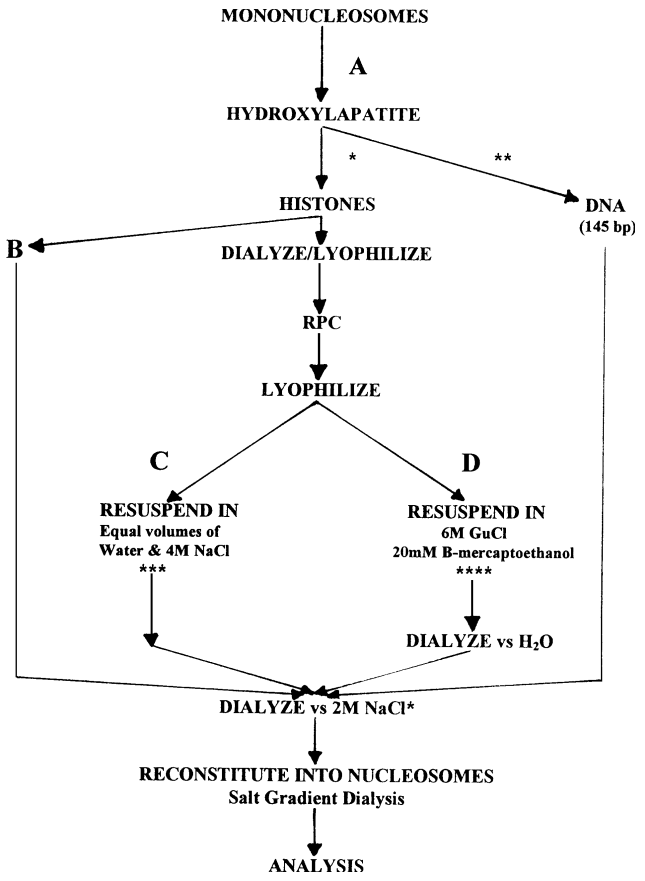

Figure 1 Flow chart summarizing the different reconstitution procedures followed in the preparation of the different reconstituted nucleosome core particle complexes, starting from hydroxyapatite/salt-extracted histones

*The core histones were eluted with 2 M NaCl/0.1 M potassium phosphate buffer (pH 6.8). **Nucleosomal DNA was eluted with 0.5 M potassium phosphate (pH 6.8) [17,44]. ***All NaCl solutions had a final buffer composition consisting of 50 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 mM DTT. ****The 6 M guanidinium chloride solution was in 50 mM Tris/HCl, pH 7.6. Lyophilize, freeze-drying; RPC, RP-HPLC; B-mercaptoethanol, β-mercaptoethanol; GuCl, guanidinium chloride.

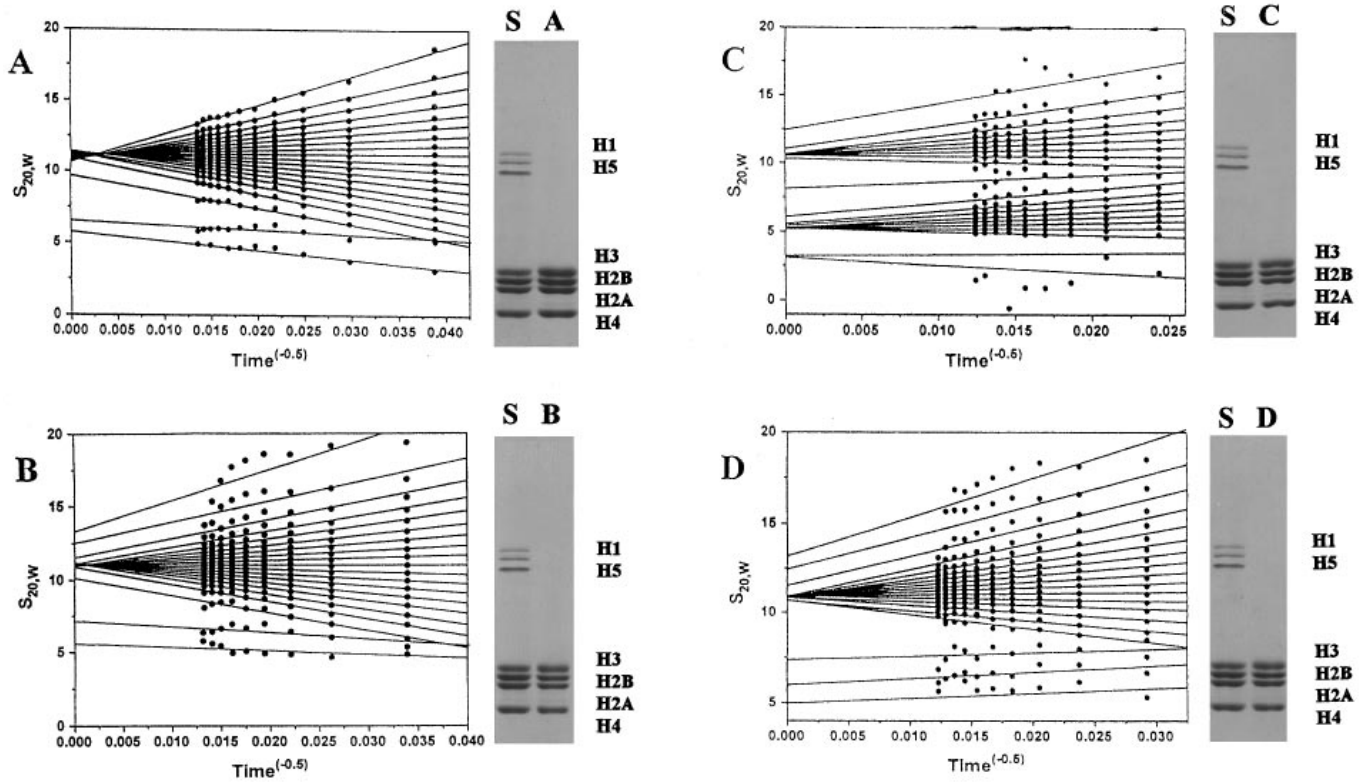

Figure 2 Sedimentation velocity analysis

(**A**) Native nucleosome core particles (fraction A, Figure 1); (**B**) nucleosome core particles reconstituted from 2 M NaCl/hydroxyapatite-purified core histones (fraction B, Figure 1); (**C**) nucleosome core particles reconstituted from RP-HPLC-purified core histones (fraction C, Figure 1); (**D**) nucleosome core particles reconstituted from RP-HPLC-purified core histones treated with guanidinium chloride (fraction D, Figure 1) (see text for more detail). The runs were performed at 20 °C and 40 000 rev./min using the buffer 0.1 M NaCl/20 mM Tris/HCl (pH 7.5)/0.5 mM EDTA. The analysis was carried out according to van Holde and Weischet [45] in which the lines converge towards a common $s_{20,w}$ (Svedbergs) value and the number of lines is proportional to the fraction of sample represented. The SDS/PAGE patterns of the corresponding core histones from the analysed complexes are shown on the right-hand sides of each Figure. S, chicken erythrocyte whole-histone standard. Time is shown in seconds.

column) (see Figure 1) and were reconstituted into nucleosome core particles by salt-gradient dialysis, and further characterized by analytical ultracentrifugation, CD and DNase I footprinting (see Figures 2–4).

Analysis of the results shown in Figures 2–4 clearly indicate that the nucleosome core particles reconstituted from RP-HPLC histones, treated with guanidinium chloride and β-mercaptoethanol (fraction D), exhibit structural characteristics that are almost indistinguishable from those of native nucleosome core particles (fraction A).

The sedimentation coefficients of the lines converging to a single point in Figure 2 were found to be 11.06, 11.07 and 10.9 ± 0.2 S for the nucleosome core particles corresponding to fractions A, B and D respectively. Fraction C shows the presence of two main populations of macromolecules sedimenting at 10.6 ± 0.2 (40%) and 5.4 ± 0.2 S (40%), the latter one corresponding to free 145-bp DNA [26]. In this fraction, as well as in fractions B and D, there is a small amount (5–15%) of material sedimenting with $S > 11$, which reflects the binding of extra histones to the nucleosome core particle [26], which is most probably due to an error in the estimation of the histone/DNA ratio. Fractions A, B and D also show a variable amount (10–15%) of < 11 S sedimenting material, which results from the partial dissociation of the nucleosome core particles, which is a result of the experimental conditions of ionic strength and temperature [26].

The hydrodynamic characteristics of the different reconstituted nucleosome core particle fractions are mirrored by the spectroscopic and nuclease accessibility features shown in Figures 3 and 4. The increase in ellipticity at 282.5 nm exhibited by fraction C (see Figure 3, curve C and also Figure 2C) can be attributed to the presence of a larger amount of free DNA [27], which also accounts for the apparent loss of protection against DNase I sensitivity observed in Figure 4 (lane C).

RP-HPLC prevents the correct association of histones into a core as a result of changes in their secondary structure

Due to the apparent inability of RP-HPLC-purified histones to fully reconstitute nucleosome core particles, we decided to compare the association behaviour of the histone fraction B with that of fractions C and D (see Figure 1).

Under the experimental conditions (see Figure 5A) native core histones sediment as a monodisperse sample, with 3.8 ± 0.2 S, in agreement with previous reports [28–31]. In contrast, RP-HPLC-purified histones (fraction C, Figure 1) exhibit a significant extent of heterogeneity (Figure 5A2), with only approx. 50% of the

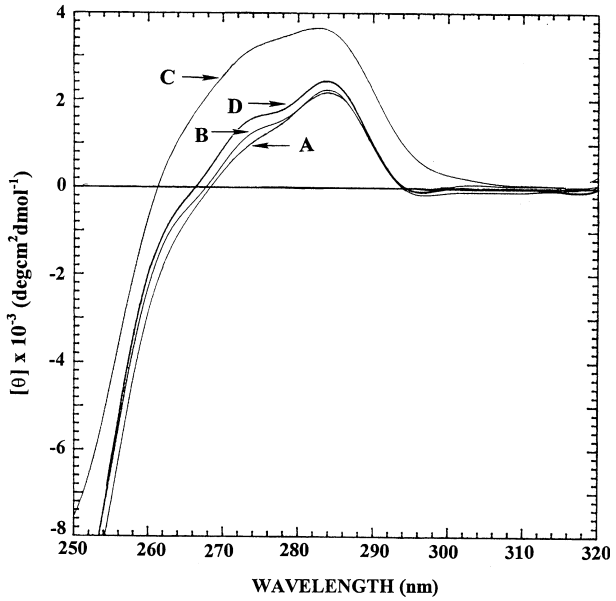

Figure 3 CD spectra of native nucleosome core particles (A), and nucleosome core particles reconstituted from histone fractions B, C and D (see Figure 1)

Ordinate shows $10^{-3} \times [\theta]$ (degrees $\cdot$ cm^2 $\cdot$ dmol^{-1}).

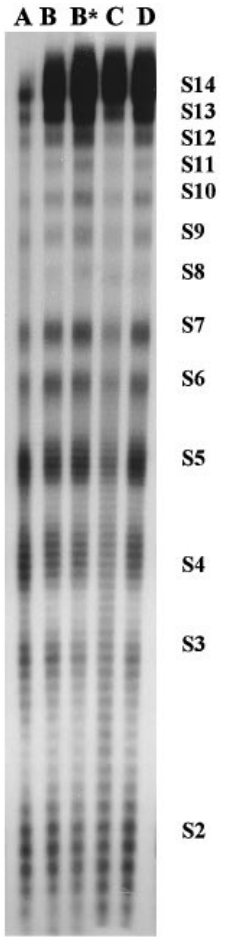

Figure 4 DNase I footprinting analysis of native nucleosome core particles (A) compared with different nucleosome core particle reconstituted complexes

The legends for histone fractions B, C and D are the same as in Figures 1 and 2. B* is the same as B, except that the core histones were frozen at −80 °C (for storage purposes) before they were used in the reconstitution experiments. In B, the hydroxyapatite-derived core histones were used for reconstitution immediately after purification. The sites of preferential DNase I cleavage are designated S1–S14 with respect to the labelled 5′-end of the nucleosome core particle [46].

sample sedimenting at 3.8 S and the rest of the sample sedimenting at lower $s_{20,w}$ values. Thus from the hydrodynamic standpoint, the RP-HPLC-purified histones exhibit an altered association that can be reverted to monodispersity when they are treated with 6 M guanidinium chloride and β-mercaptoethanol (fraction D, results not shown).

Next we decided to analyse the effect of RP-HPLC on the secondary structure of the histones. The results are shown in Figure 5(B). The CD spectrum of the native histone octamers is almost identical with that reported previously [32]. RP-HPLC-purified histones exhibit a similar spectrum, but the molar ellipticities at 222 and 208 nm have been significantly reduced. Since it is possible to estimate the α-helical contribution to the spectrum from the ellipticity at 222 nm [33], we estimated that the reduction on the ellipticity at 222 nm represents a decrease of 15 % in the overall α-helix context of these histones. These alterations in the secondary structure are most probably responsible for the anomalous association pattern of these histones.

Effects of the extraction method and biological source of the histones on nucleosome core particle reconstitution

Once we realized that it was possible to reverse the deleterious effects that RP-HPLC fractionation has on the structure and association of histones, we decided to find out if the extraction method, or the biological source of histones, could have any further effects.

Figure 6 (lanes 2, 4 and 6) shows the compositional characteristics of 0.4 M HCl-extracted histones from chicken, lamprey and alligator used in this analysis. The 0.4 M HCl extracts were RP-HPLC fractionated. The different core histone fractions were then mixed in stoichiometric amounts (Figure 6, lanes 3, 5 and 7) and the resulting protein mixture was reconstituted by guanidinium chloride treatment, as described earlier.

When Figures 7(A) and 7(B) are compared with the results shown in Figures 2 and 4 (with the exception of the lamprey, Figure 7A3), neither acid extraction nor the source of the histones appear to have any additional effect on the ability of guanidinium chloride to reverse the structural damage introduced by RP-HPLC fractionation.

In the case of lamprey histones, Figure 7(A3) shows that although approx. 60–70 % of the reconstituted nucleosomes sediment with an $s_{20,w}$ of 10.8 ± 0.2 S, which corresponds to nucleosome core particles, 30 % of the sample sediments as free DNA (5.7 ± 0.2 S). The fraction sedimenting as 5.7 S corresponds to naked DNA, as corroborated by native PAGE (results not shown). At this point it is not clear if this behaviour is inherent to the intrinsic properties of the lamprey histones which lead to nucleosomes with a slightly decreased stability under the ionic conditions at which the samples were analysed (100 mM NaCl) [26], or to problems that could have arisen during the reconstitution procedure or handling of the sample before its analysis.

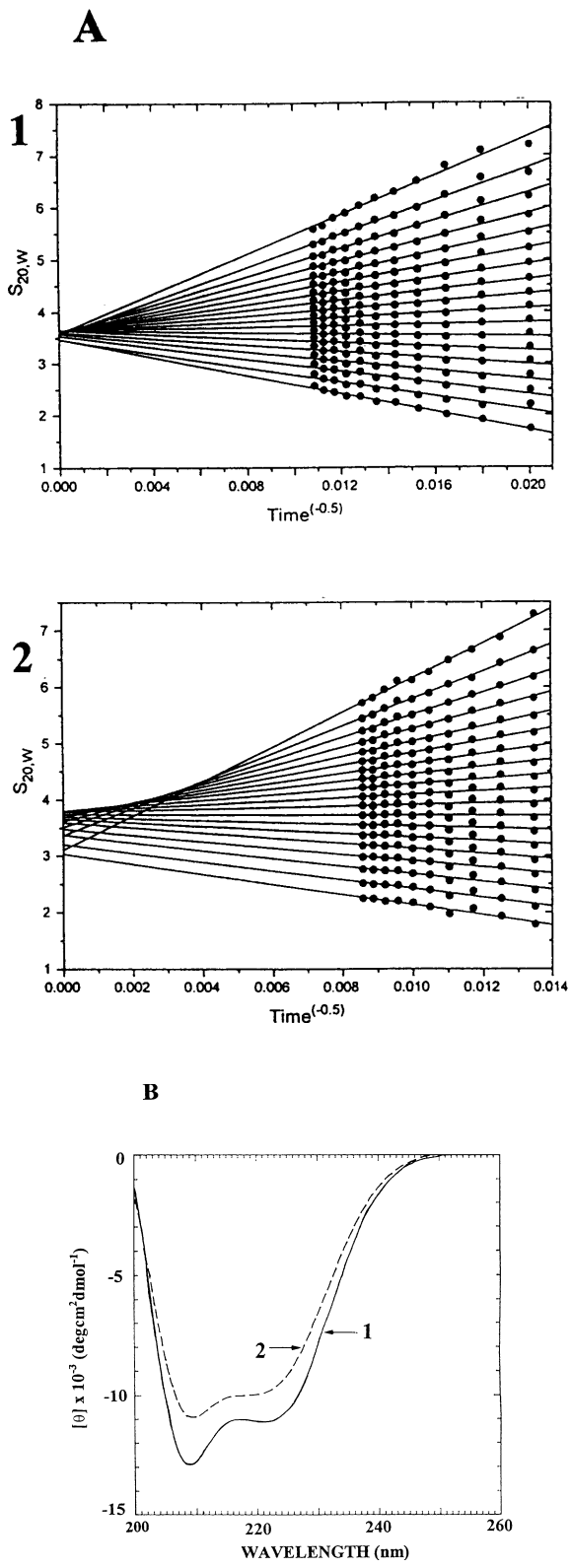

Figure 5 (A) Sedimentation velocity and (B) CD analysis of: (1) core histone fraction B; and (2) core histone fraction C (see Figure 1)

For the CD analysis, the samples were dialysed against 10 mM sodium phosphate buffer, pH 7.2. For the sedimentation velocity analysis the samples (at a concentration of approx. 8 mg/ml) were dialysed against 2 M NaCl/10 mM Tris/HCl/1 mM EDTA (pH 7.5). The runs were carried out at 20 °C and 44000 rev./min. (**B**) Ordinate shows $10^{-3} \times [\theta]$ (degrees $\cdot$ cm^2 $\cdot$ dmol^{-1}). $s_{20,w}$ represents Svedbergs; time is shown in seconds.

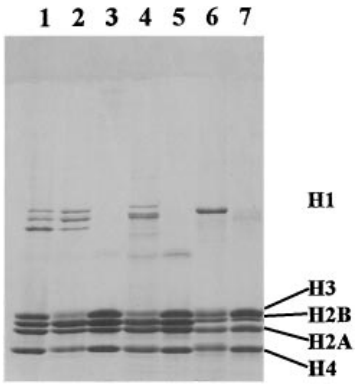

Figure 6 SDS/PAGE of acid-extracted histones

The histones (H1–H4) were extracted by using 0.4 M HCl, from: lanes 2 and 3, chicken erythrocyte; lanes 4 and 5, alligator; lanes 6 and 7, lamprey; before (lanes 2, 4 and 6) and after (lanes 3, 5 and 7) RP-HPLC purification. Lane 1, chicken erythrocyte histone standard.

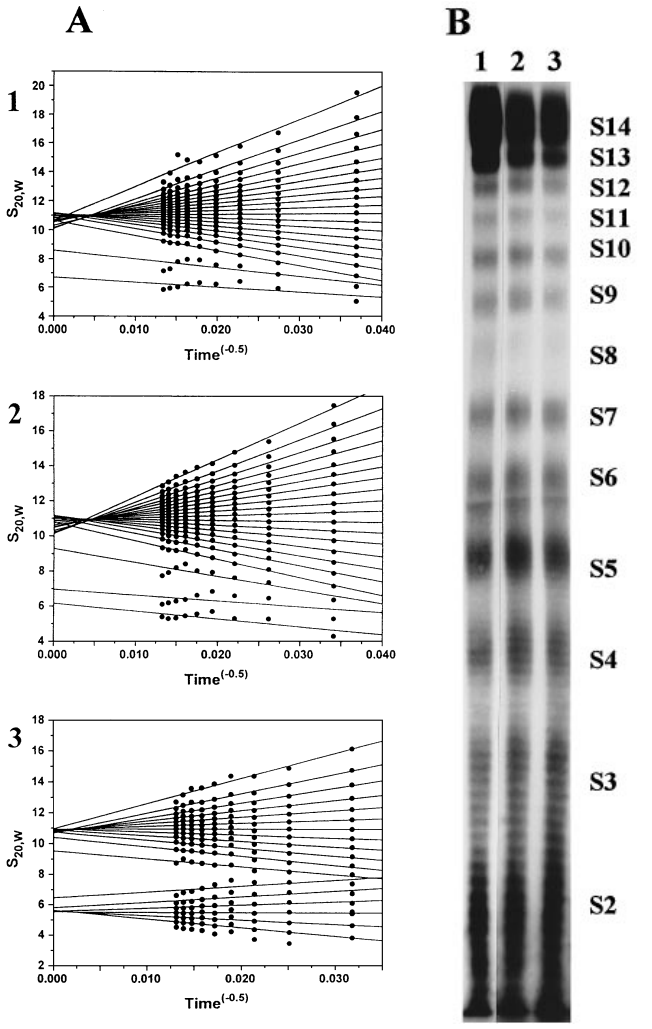

Figure 7 (A) Sedimentation velocity analysis and (B) DNase I footprinting analysis of RP-HPLC-purified core histones from (1) chicken erythrocyte, (2) alligator and (3) lamprey reconstituted nucleosomes with 0.4 M HCl histone extracts

The sites of preferential DNase I cleavage are designated S1–S14 with respect to the labelled 5′-end of the nucleosome core particle [46]. In (A), $s_{20,w}$ represents Svedbergs; time is shown in seconds.

DISCUSSION

The results presented conclusively show that it is possible to use RP-HPLC-fractionated histones to reconstitute nucleosome core particle complexes with native-like characteristics or at least with the same native-like characteristics that can be obtained upon reconstitution with salt-extracted histones (compare B and D in Figures 2–4).

We also found that the reconstitution results obtained with the RP-HPLC-fractionated histones that had been reconstituted with guanidinium chloride (see Figure 1D) were independent of whether or not the trifluoroacetic acid of the RP-HPLC fractions had been neutralized [34] (with NaOH) before freeze-drying (results not shown). This suggests that the major damaging effect of RP-HPLC on the structure of histones is due to the high acetonitrile concentrations of the mobile phase. Most probably this happens through an alteration of the secondary structure of histones (Figure 5B) which ultimately affects their association properties (Figure 5A).

The ability of acid-extracted histones to reconstitute into native-like chromatin complexes has been a controversial issue [35]. However, our results show that it is indeed possible to reconstitute both histone octamers and native-like nucleosome core particles using acid-extracted proteins [24,36,37].

The availability of a method to reconstitute chromatin complexes from RP-HPLC histones is timely and important. It should provide an extremely useful method for the reconstitution of chromatin complexes starting from histone variants or from post-translationally modified histones (acetylated, methylated, phosphorylated or ubiquitinated) [38], which are usually present in small amounts. RP-HPLC can, for instance, resolve the H2A.2 and H2A.1 variants [4] and can be used in conjunction with other HPLC techniques to purify histone fractions with a well-defined extent of acetylation [39]. The histone fractions thus obtained could be combined with sequence-defined DNA templates to further enhance the powerful potential of the reconstituted chromatin complexes that are currently used for the analysis of chromatin structure [17,40] and function [41–43].

We are very indebted to Professor Harold Kasinsky from the Zoology Department at the University of British Columbia and to Dr. Ruth M. Elsey from the Department of Wildlife and Fisheries at the Rockefeller Wildlife Refuge in Louisiana, for providing us with the lamprey and alligator testes. We are also very grateful to Maree Roome for her skilful typing of the manuscript. This work was supported by an operating grant of the Medical Research Council of Canada (MR 13104) to J.A.

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Received 27 May 1997/14 July 1997; accepted 17 July 1997