ELSEVIER

Journal of Chromatography B, 744 (2000) 33–46

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Resolution of allelic and non-allelic variants of histone H1 by cation-exchange-hydrophilic-interaction chromatography

Craig A. Mizzen[a,*], Andrew J. Alpert[b], Lyne Lévesque[a], Theo. P.A. Kruck[a], Donald R. McLachlan[a]

[a]*Centre for Research in Neurodegenerative Diseases, University of Toronto, Toronto, Ontario, Canada M5S 1A8*
[b]*PolyLC Inc., 9151 Rumsey Road, Suite 180, Columbia, MD 21045, USA*

Received 8 November 1999; received in revised form 14 March 2000; accepted 23 March 2000

Abstract

A mixed-mode high-performance liquid chromatography (HPLC) method that resolves the six known non-allelic variants of chicken erythrocyte histone H1 is described. Common, but previously unknown, allelic variants of H1 that comigrate in polyacrylamide gel electrophoresis are also resolved. The resolution of H1 variants achieved by this method should be useful in determining the functional significance of H1 sequence heterogeneity and in analyses of post-translational modification of H1. Furthermore, the principles behind the separation should be applicable to analyses of polymorphism in other proteins.

Keywords: Histone H1

1. Introduction

An octamer of the core histones, H2A, H2B, H3 and H4, associates with DNA to form the basic structural unit of eukaryotic chromatin, the nucleosome [1]. A fifth type of histone, referred to as linker or H1 histone, binds the exterior surface of nucleosomes and portions of the linker DNA joining adjacent nucleosomes. Recent advances in biochemistry and genetics have established primary roles for core histones, and their post-translational modification, in mechanisms that regulate chromatin transcription [2,3], but the roles of linker histones in nuclear function are less defined. Early analyses suggested that H1 was a general repressor of transcription in vitro [4], however, recent investigations have shown that, in vivo, these proteins can regulate transcription positively or negatively in a gene-specific fashion [5] and contribute to the supranucleosomal folding of chromatin [6]. Additionally, the correlation of mitotic hyperphosphorylation of H1 with metaphase chromosome condensation suggests H1 phosphorylation promotes chromatin condensation [7], but other evidence supports the possibility that phosphorylation of H1 is associated with chromatin decondensation [8] and enhanced transcription [9,10].

Although the primary structures of all the histones are, in general, highly conserved across plant and animal phyla, copies of genes arising by duplication

*Corresponding author. Present address: Department of Biochemistry and Molecular Genetics, University of Virginia Health Sciences Center, 6222 Jordan Hall, 1300 Jefferson Park Ave., P.O. Box 800733, Charlottesville, VA 22908-0733, USA. Tel.: +1-804-982-1774; fax: +1-804-924-5069.

E-mail address: cam8y@virginia.edu (C.A. Mizzen)

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00210-3

have diverged during evolution such that multiple genes, encoding variants (subtypes) with limited differences in amino acid sequence, exist for nearly all the histones in most eukaryotes [1,11–13]. The H1 group is the most heterogeneous of all the histones and a long-standing question regarding the role(s) of H1 in chromatin is whether H1 variants have evolved to perform functionally distinct roles [1,11,12,14]. However, the limited resolution of H1 variants provided by existing methods has hindered comprehensive structural and functional analyses of these proteins. Cation-exchange chromatography on Bio-Rex 70 (Amberlite IRC-50) [15] and two-dimensional electrophoresis [16] have been used most frequently to resolve forms of H1. Alterations in the abundance of the forms resolved by these methods correlate with cellular differentiation and cell cycle progression (e.g. [15,17,18]), supporting the notion of functional diversity for at least some H1 variants. However, incomplete characterization of the components resolved and, in some cases, the apparent heterogeneous nature of the fractions resolved, complicate interpretation of these studies.

More recently, high-performance liquid chromatography (HPLC) techniques have been applied to resolve H1 variants. To date, the majority of reports have described the use of reversed-phase HPLC (RP-HPLC). However, the resolution of H1 variants by RP-HPLC is often lower than that achieved by electrophoretic methods. In some reports, a single peak was observed upon RP-HPLC of H1 samples exhibiting electrophoretic heterogeneity [19–23], although two to five peaks were resolved in other cases [24–36]. Even though the volatile mobile phases routinely employed in RP-HPLC facilitate subsequent biomolecular characterization, the limited analyses performed in many of these studies make it difficult to assess whether any H1 variants were purified to homogeneity. Frequently, one or more of the H1 peaks isolated by RP-HPLC were shown to be heterogeneous in electrophoresis [20,21,23,25, 28,29,32,33,35–38].

We have examined the ability of cation-exchange-HPLC (CX-HPLC) to resolve H1 variants using chicken erythrocyte H1 as a test sample. Six genes, estimated to represent the total H1 gene complement in this species, have been isolated and sequenced [39]. Moreover, the six protein bands resolved by acetic acid–urea-polyacrylamide gel electrophoresis (AU-PAGE) of chicken erythrocyte H1 have been aligned with these sequences [40]. Conventional CX-HPLC did not completely resolve any of these H1 variants. However, based on work described previously [41,42], we developed a mixed-mode method using a hydrophilic weak cation-exchange stationary phase with largely non-aqueous mobile phases that resolved all six known chicken H1 variants. Additionally, multiple peaks were resolved for some of these proteins and we present evidence that they represent allelic variants of known H1 genes. The term hydrophilic-interaction chromatography (HILIC) has been proposed to describe separations of solutes in hydrophobic mobile phases using polar stationary phases [43], and numerous substances have been separated on ionizable and neutral supports under conditions in which polar or hydrophilic interactions are thought to predominate [38,43–47]. Because both electrostatic and hydrophilic interactions appear to contribute significantly to the resolution of H1 variants under our conditions, we acknowledge the mixed-mode nature of the technique by referring to it as cation-exchange-hydrophilic-interaction chromatography (CX-HILIC), and show here that solvent-induced alterations in the secondary structure of H1 also appear to be involved. CX-HILIC reveals that chicken erythrocyte H1 is much more heterogeneous than has been reported previously, and our data suggest this is attributable to the existence of allelic variants that are not resolved by other techniques. CX-HILIC and related methods appear to be particularly well-suited for the resolution of polypeptides that differ only slightly in their physical and chemical properties and hence are not resolved by conventional HPLC techniques.

2. Materials and methods

2.1. Chemicals

Trifluoroacetic acid (TFA) was from Pierce (Sequanal grade, Rockford, IL, USA). HPLC-grade sodium perchlorate and acetonitrile were from Fisher Scientific (Fairlawn, NJ, USA) and VWR Scientific (Chester, PA, USA), respectively. Acrylamide, bis-acrylamide and sodium dodecyl sulfate (SDS) were

from Bio-Rad (Hercules, CA, USA). Water was prepared with a Milli-Q deionization system (Millipore, Bedford, MA, USA). All other chemicals employed were ACS grade or better.

2.2. Isolation of chicken erythrocyte nuclei

Pooled chicken blood, comprised of approximately 10 ml of blood from each of 100 adult animals, was obtained at a local poultry packer. Blood was collected into an equal volume of ice-cold 154 m*M* NaCl, 20 m*M* Na–EDTA pH 8.0, 1.0 m*M* phenylmethylsulfonyl fluoride (PMSF) and subsequently pooled with other samples on ice. Blood from single chickens was collected by perfusion of anesthetized birds with heparinized saline. Nuclei were prepared from pooled and single animal blood samples by detergent lysis of erythrocytes as described previously [48].

2.3. Extraction of crude H1–H5

2.3.1. Acid extraction

All steps were performed at 4°C. Total histone was extracted from freshly prepared nuclei with 0.4 *N* H_2SO_4 and core histones selectively precipitated by the addition of perchloric acid (PCA, 5% (w/v) final). The crude H1–H5 mixture was recovered from the 5% PCA supernatant by trichloroacetic acid (TCA) precipitation (20% (w/v) final). Protein precipitates were washed twice with acetone containing 0.1% (v/v) HCl, twice with acetone, dried under vacuum and stored dry at −70°C.

2.3.2. Salt extraction

Alternatively, crude H1–H5 was selectively extracted from pooled erythrocyte nuclei with 0.6 *M* NaCl containing 0.5 m*M* PMSF at 4°C. Following clarification by centrifugation, the supernatant was dialysed against 150 m*M* NaCl, 0.5 m*M* PMSF, 10 m*M* sodium phosphate pH 7.0 and stored as a frozen solution at −70°C.

2.4. Isolation of total H1 from crude H1–H5

2.4.1. Large scale preparation

A large sample of electrophoretically pure total H1 for HPLC method development (i.e. containing all H1 forms present in the crude extract but not detectable amounts of H5 or other proteins) was prepared by gel filtration of crude H1–H5 obtained from pooled erythrocyte nuclei by acid-extraction. Two columns (5.0 cm I.D.×100 cm) packed with Bio-Gel P100 (100–200 mesh; Bio-Rad, Hercules, CA, USA) were eluted in series at 50 ml/h with 10 m*M* HCl and 3 m*M* NaN_3 at 22°C. Elution was monitored at 218 nm. Fractions were analysed by gel electrophoresis and those containing only H1 were pooled and the protein recovered by TCA precipitation.

2.4.2. Small scale preparation

Total H1 was prepared from the blood of single chickens by acid-extraction of isolated nuclei followed by RP-HPLC as described below. Total H1 was prepared from salt extracts of pooled erythrocyte nuclei by chromatography on Sephadex CMC-25 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as described previously [49].

2.5. Polyacrylamide gel electrophoresis

Electrophoresis in 12% and 15% (w/v) polyacrylamide gels containing SDS (SDS-PAGE) was performed according to Laemmli [50]. Electrophoresis in 50 cm long 15% (w/v) polyacrylamide gels containing 2.5 *M* urea and 0.9 *N* acetic acid (AU-PAGE) was performed as described by Lennox and Cohen [16]. Gels were stained with Coomassie Blue R-250 or silver nitrate [51].

2.6. HPLC

Separations were performed at room temperature on Waters (Bedford, MA, USA) or Beckman (Fullerton, CA, USA) binary gradient chromatographs with computerized data collection and instrument control. Protein elution was monitored at 214 nm.

2.6.1. RP-HPLC

RP-HPLC was performed using a Chromegabond MC-18 column (4.6 mm I.D.×250 mm, 5-μ diameter silica particles with 30-nm diameter pores, E.S. Industries, Berlin, NJ, USA) eluted with a linear gradient of 5–60% (v/v) acetonitrile in 0.1% (v/v) TFA over 2 h at 0.8 ml/min. Proteins were re-

covered from RP-HPLC eluents by solvent evaporation using a Speed-Vac apparatus (Savant Inc., Farmingdale, NY, USA).

2.6.2. CX-HPLC

The following CX-HPLC columns were tested for their ability to resolve pooled total erythrocyte H1 using comparable linear gradients of sodium chloride in 20 m*M* sodium phosphate pH 6.5: Mono-S (5.0 mm I.D.×50 mm; Amersham Pharmacia Biotech, Piscataway, NJ, USA), Protein-Pak SP-5PW (7.0 mm I.D.×50 mm; Waters, Bedford, MA, USA), Tessek Hema-Bio 1000CM (5.0 mm I.D.×50 mm; Fisher Scientific, Fairlawn, NJ, USA), Synchropak CM-300 (4.6 mm I.D.×250 mm; Synchrom, Lafayette, IN, USA), Spherogel TSK CM3SW and Spherogel TSK IEX-535CM (4.6 mm I.D.×250 mm; Beckman, Fullerton, CA, USA), Aquapore CX-300 (4.6 mm I.D.×250 mm; Applied Biosystems, Foster City, CA, USA) PolySULFOETHYL A and PolyCAT A (4.6 mm I.D.×200 mm; PolyLC Inc., Columbia, MD, USA). Of these, PolyCAT A resolved the greatest number of peaks and was selected for further method development.

2.6.3. CX-HILIC

CX-HILIC was performed on PolyCAT A columns (4.6 mm I.D.×200 mm) packed with 5-μ diameter particles with various pore diameters as described in the figure legends. CX-HPLC buffers containing acetonitrile were prepared by dissolving all components except water in the desired volume of acetonitrile using a magnetic stirrer. Water was then added to bring the volume to 95% of the intended final volume and the solution allowed to come to room temperature (22°C). The pH was adjusted using a glass combination electrode and then water was added to give the final intended volume. This method of preparation was adopted to avoid errors due to the effects of organic solvents on buffer ionization constants. In the presence of more than 50% (v/v) acetonitrile, the apparent pK_a of phosphate and propionate buffers were shifted upwards such that the buffering capacity of 10 m*M* sodium phosphate was less than that of sodium propionate at pH 6.5. Chromatograms obtained using phosphate or propionate buffers in 70% (v/v) acetonitrile were identical but retention time reproducibility was greater with the propionate system. Buffers were vacuum-filtered using a 0.45-μ pore diameter Durapore membrane (Millipore, Bedford, MA, USA) prior to use. Proteins were recovered from CX-HILIC eluents by TCA precipitation after evaporation of acetonitrile under vacuum in a Speed-Vac apparatus.

2.7. Circular dichroism spectroscopy

CD spectra were recorded on a J720 spectropolarimeter (JASCO, Easton, MD, USA) using a 0.1-mm pathlength quartz cell. Identical samples (80 μg) of pooled total erythrocyte H1 dried in microcentrifuge tubes were dissolved in 10 μl of water and then brought to a final volume of 200 μl with the appropriate solvents several hours prior to measurement. Ten scans were averaged for each sample.

3. Results

3.1. Resolution of chicken erythrocyte H1 variants by RP, CX-HPLC and CX-HILIC

Total H1 from pooled blood, comprising six bands in AU-PAGE (e.g. Fig. 6), eluted as a single peak in RP-HPLC even though a shallow gradient was employed (Fig. 1A). Similar results were obtained using RP-HPLC columns from different manufacturers using a variety of elution strategies (data not shown). In contrast, two or more forms of H1 were partially resolved when silica-based and polymer-based CX-HPLC columns from several manufacturers were eluted with linear gradients of NaCl in aqueous buffer at pH 6.5 (data not shown). Under these test conditions, columns packed with PolyCAT A provided the greatest resolution of chicken H1 variants (data not shown). We found that eluent composition significantly affected the retention and resolution of H1 on PolyCAT A. As shown in Fig. 1B and 1D, when the same $NaClO_4$ gradient was employed with buffers containing 0, 40 or 70% (v/v) acetonitrile, the retention of H1 was lower in buffers containing 40% (v/v) acetonitrile. The inset of Fig. 1C shows a separation in 40% (v/v) acetonitrile using a $NaClO_4$ gradient with the same slope as those in Fig. 1B and 1D, but after reduction of the initial and final salt concentrations to make the

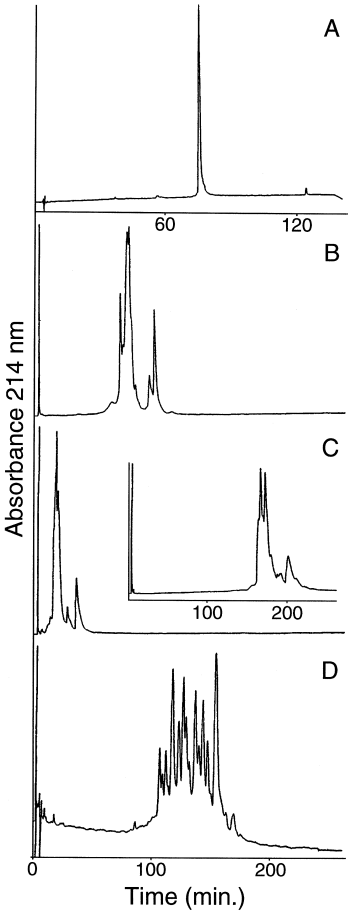

Fig. 1. Resolution of H1 variants by RP-HPLC, CX-HPLC and CX-HILIC. Identical 100-μg samples of pooled chicken erythrocyte H1 were chromatographed by (A) RP-HPLC, (B and C) CX-HPLC and (D) CX-HILIC. (A) A Chromegabond MC-18 column was eluted with a 2-h linear gradient from 5 to 60% (v/v) acetonitrile in 0.1% (v/v) TFA. (B–D) A PolyCAT A column (100-nm diameter pores) was eluted with a 4-h linear gradient from 380 to 590 m*M* $NaClO_4$. Buffers were (B) 10 m*M* sodium phosphate pH 6.5, (C) 10 m*M* sodium phosphate pH 6.5 containing 40% (v/v) acetonitrile and (D) 10 m*M* propionic acid pH 6.5 containing 70% (v/v) acetonitrile. The profile obtained with a 4-h linear gradient from 230 to 440 m*M* $NaClO_4$ in 10 m*M* sodium phosphate pH 6.5 containing 40% (v/v) acetonitrile is shown in the inset of (C). In all panels (A–D), the flow-rate was 0.8 ml/min and detection was at 214 nm.

overall retention of H1 similar to that in Fig. 1B–D. Despite this adjustment, the resolution of H1 variants in 40% (v/v) acetonitrile was similar or slightly inferior to that achieved in completely aqueous buffer. In contrast, the resolution of H1 variants in buffer containing 70% (v/v) acetonitrile (Fig. 1D) was markedly greater than that achieved in buffers containing less acetonitrile. In the absence of acetonitrile, various gradient materials, including potassium chloride, sodium chloride and sodium sulfate gave results similar to those shown for sodium perchlorate in Fig. 1B (data not shown). However, sodium perchlorate was the only salt available in sufficiently pure form that we found to be compatible with chromatography in buffers composed primarily of acetonitrile. Detailed optimization experiments established that the retention and resolution of H1 variants were decreased when buffers containing 70% (v/v) acetonitrile were adjusted to pH values other than 6.5 (data not shown). In accordance with the rationale discussed below, we designate cation-exchange chromatography performed on a hydrophilic support, such as PolyCAT A with largely non-aqueous mobile phases, as in Fig. 1D, by the acronym CX-HILIC.

3.2. Effect of acetonitrile on H1 retention in CX-HPLC

To better understand the effect of acetonitrile on the retention of chicken H1 by PolyCAT A, we performed a series of separations using identical $NaClO_4$ gradients, in which buffer acetonitrile content was increased by increments of 5 or 10% (v/v) and plotted the retention time of the earliest eluting H1 peak as a function of buffer acetonitrile content (Fig. 2). H1 retention decreased gradually as buffer acetonitrile content was increased from 0 to 40% (v/v), reached a minimum at approximately 40% (v/v) acetonitrile, increased gradually as acetonitrile content was raised from 40 to 60% (v/v), and then increased steeply between 60 and 70% (v/v) acetonitrile. Even though total chicken H1 remained soluble at a concentration of 1 mg/ml in buffers containing 90% (v/v) acetonitrile and 0.4 *M* $NaClO_4$, elution of H1 was not observed when acetonitrile concentrations greater than 72% (v/v) were employed, apparently due to precipitation of H1 within the HPLC system following injection (data not shown). Except for the spectroscopic studies described below, propionate buffer was employed in place of phosphate at acetonitrile concentrations greater than 50% (v/v) throughout this work due to the effect of high

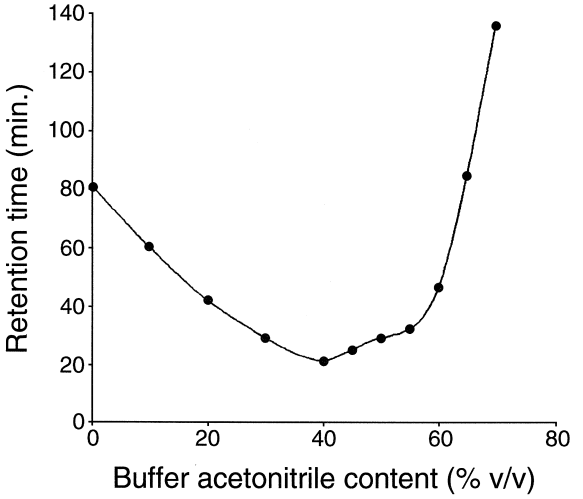

Fig. 2. Effect of acetonitrile concentration on H1 retention by PolyCAT A. Identical 100-μg samples of pooled chicken erythrocyte H1 were chromatographed on the same PolyCAT A column (100-nm diameter pores) with a 4-h gradient from 380 to 590 m*M* $NaClO_4$ in mobile phases containing 0, 10, 20, 30, 40, 45, 50, 55, 60, 65 and 70% (v/v) acetonitrile. Buffers were 10 m*M* sodium phosphate pH 6.5 for mobile phases containing 0–50% (v/v) acetonitrile and 10 m*M* propionic acid pH 6.5 for mobile phases containing 55–70% (v/v) acetonitrile. All other conditions were as in Fig. 1. The retention time of the first H1 component to elute is plotted as a function of the mobile phase acetonitrile concentration.

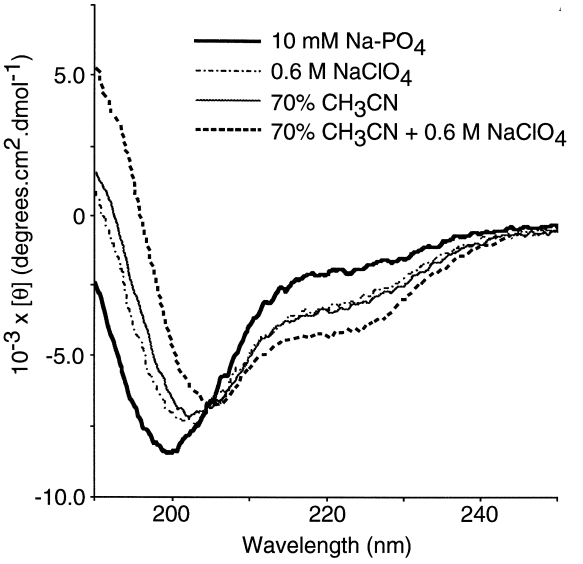

Fig. 3. Effect of acetonitrile and sodium perchlorate on H1 secondary structure. CD spectra are shown of identical samples of pooled chicken erythrocyte H1 dissolved in 10 m*M* sodium phosphate pH 6.5, 10 m*M* sodium phosphate pH 6.5 containing 0.6 *M* $NaClO_4$, 10 m*M* sodium phosphate pH 6.5 containing 70% (v/v) acetonitrile and 10 m*M* sodium phosphate pH 6.5 containing 0.6 *M* $NaClO_4$ and 70% (v/v) acetonitrile.

concentrations of organic solvent on buffer pK_a (see Section 2.6.3).

3.3. H1 conformation in buffers containing CH_3CN and $NaClO_4$

To ascertain whether alterations in H1 conformation contributed to the resolution of variants achieved in buffers containing 70% (v/v) acetonitrile, we studied the effects of acetonitrile and sodium perchlorate on the conformation of total H1 using circular dichroism (CD) spectroscopy (Fig. 3). $NaClO_4$ (0.6 *M*) and 70% (v/v) CH_3CN each increased the α-helix content of total H1 to a similar extent (enhanced negative ellipticity at 222 nm) relative to its conformation in 10 m*M* sodium phosphate (pH 6.5) alone. When acetonitrile and sodium perchlorate were used in combination, i.e. as in CX-HILIC, the effects were additive. Identical results were obtained for solutions containing propionate in place of phosphate (data not shown). Thomas and colleagues [52,53] have shown previously that 1 *M* $NaClO_4$ and 50–65% (v/v) 2,2,2-trifluoroethanol (TFE) stabilize helices in chicken H1.

3.4. Effect of HPLC support pore diameter

The pore size of the PolyCAT A packing had a significant effect on H1 retention and variant resolution. Using gradients that were 2 h long and had identical slopes, we compared the resolution of H1 variants on PolyCAT A packings prepared using silicas with average pore diameters of 20, 30, 100 and 400 nm (according to manufacturers specifications) under CX-HPLC (Fig. 4A–D, 0% (v/v) acetonitrile) and CX-HILIC conditions (Fig. 4E–H, 70% (v/v) acetonitrile). The overall retention of H1 decreased with increasing support pore diameter under both CX-HPLC and CX-HILIC conditions. In aqueous buffer, H1 variants were best resolved on the 30-nm diameter pore support (Fig. 4B), but this

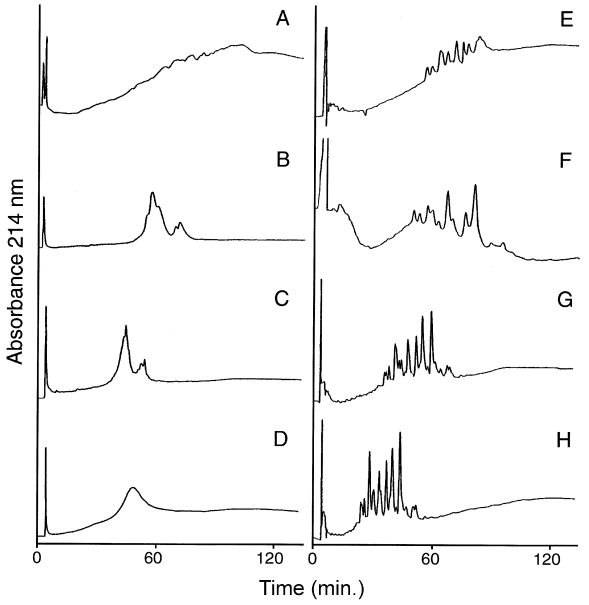

Fig. 4. Resolution of H1 variants by CX-HPLC and CX-HILIC on PolyCAT A supports with different pore diameters. Identical 100-μg samples of pooled chicken erythrocyte H1 were chromatographed on PolyCAT A packings with (A and E) 20, (B and F) 30, (C and G) 100 and (D and H) 400-nm diameter pores. Two-hour linear gradients were employed (A–D) from 400 to 600 m*M* $NaClO_4$ in 10 m*M* sodium phosphate pH 6.5 or (E–H) from 500 to 700 m*M* $NaClO_4$ in 10 m*M* propionic acid pH 6.5 containing 70% (v/v) acetonitrile. All other conditions were as in Fig. 1.

was inferior to the resolution achieved with any of the four different pore diameter supports using CX-HILIC conditions (Fig. 4E–H). Under the conditions employed in this comparison, similar numbers of variants were resolved by CX-HILIC on the 100 and 400 nm diameter pore packings (Fig. 4G and H), but the degree of resolution of some components was greater on the 100-nm diameter pore support. Additionally, careful comparison of the numbers, shapes and spacing of the peaks resolved on the various supports suggested the possibility that different sets of variants were resolved on the 30-nm and 100-nm diameter pore supports (Fig. 4F and G).

3.5. Optimized resolution of H1 variants by CX-HILIC

Optimized CX-HILIC separations of total H1 from pooled blood on PolyCAT A packings with 30 or 100 nm diameter pores are shown in Fig. 5A and B, respectively. We found that doubling the gradient time relative to that used in Fig. 4 (i.e., increase the concentration of $NaClO_4$ by 200 m*M* linearly over 4 h) provided the greatest resolution of H1 variants on both supports. Under these conditions, the resolution of variants appeared to be similar on both columns: 11 and 12 major peaks were resolved on the 30 and

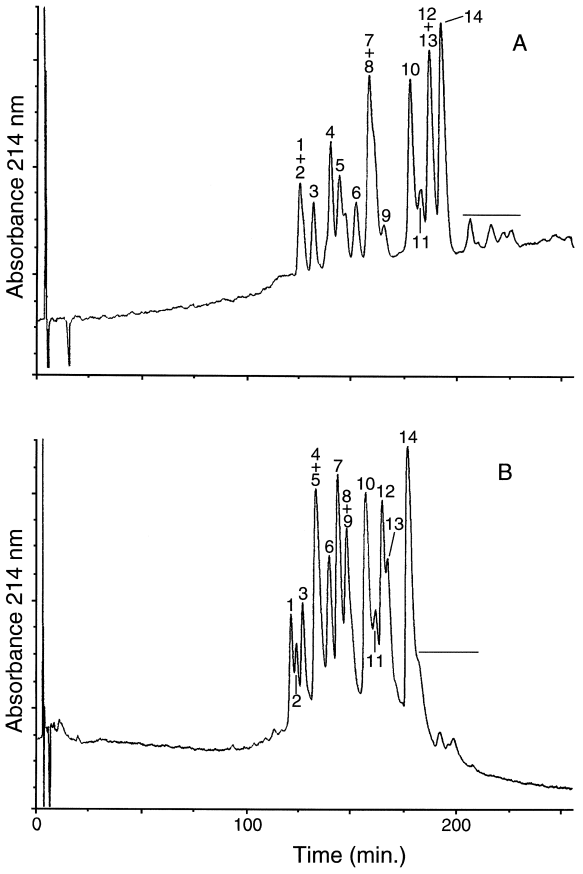

Fig. 5. Optimized resolution of H1 variants by CX-HILIC on 30 and 100-nm diameter pore PolyCAT A. Identical 100-μg samples of pooled chicken erythrocyte H1 were chromatographed on PolyCAT A supports with (A) 30 and (B) 100-nm diameter pores. Four-hour linear gradients from (A) 460 to 660 m*M* $NaClO_4$ and (B) 420 to 620 m*M* $NaClO_4$ in 10 m*M* propionic acid pH 6.5 containing 70% (v/v) acetonitrile were used. All other conditions were as in Fig. 1. Peaks are numbered according to order of elution, the same numbering is used for the AU-PAGE analysis shown in Fig. 6. The differences in resolution between the two supports were confirmed by rechromatography and PAGE analyses. Horizontal bars indicate minor components confirmed to be H1 variants by PAGE that are not characterized here.

100 nm diameter pore supports, respectively. However, electrophoretic analyses, and analyses of peaks resolved on one support by rechromatography on the other, revealed differences in the resolution of particular H1 variants on the two supports. These differences are annotated in the peak numbering used in Fig. 5. H1 variants 1 and 2, variants 7 and 8, and variants 12 and 13, comprising the first, sixth and tenth major peaks, respectively, to elute from the 30-nm diameter pore support, were resolved on the 100-nm diameter pore support. Conversely, variants 4 and 5, and variants 8 and 9, which comprised the fourth and seventh major peaks, respectively, to elute from the 100-nm diameter pore support were resolved on the 30-nm diameter pore support. Additionally, several minor H1 variants (indicated by the horizontal bars) appeared to be better resolved on the 30-nm pore support.

3.6. Identification of H1 variants resolved by CX-HILIC

Conventional SDS-PAGE resolves only two bands for chicken erythrocyte H1. However, electrophoresis in long gels containing acetic acid and urea (AU-PAGE) resolves six bands, a, a′, b, c, c′ and d, that have been shown to correspond to the six H1 genes characterized in chicken erythrocytes [40]. To identify the major variants resolved by CX-HILIC, we separated total H1 from pooled blood on 100-nm diameter pore PolyCAT A as in Fig. 5, and then resolved variants 4 and 5, and variants 8 and 9, which comprised the fourth and seventh peaks, respectively, from this initial step by CX-HILIC on 30-nm diameter pore PolyCAT A. As shown in Fig. 6, each variant separated by this procedure (designated by the same numbers used in Fig. 5) corresponded to one of the six bands comprising total H1 (lanes S) in AU-PAGE. The apparent identity of each PolyCAT A peak according to AU-PAGE mobility is listed in Table 1. Remarkably, multiple forms of H1a, H1b and H1c were resolved by CX-HILIC. Peaks 1 through 4 all migrated equivalently to H1b, peaks 7, 9, 10 and 12 migrated equivalently to H1a and peaks 5, 6 and 8 migrated equivalently to H1c (Fig. 6 and Table 1). Of the three forms of H1c (lanes 5, 6 and 8 in Fig. 6), two coeluted with forms of H1b and H1a (i.e., variants 4 and 9, respectively), in the fourth and seventh peaks to elute from 100-nm diameter pore PolyCAT A (Fig. 5). AU-PAGE analyses of the late-eluting minor components (bars in Fig. 5) suggested that additional forms of H1a′

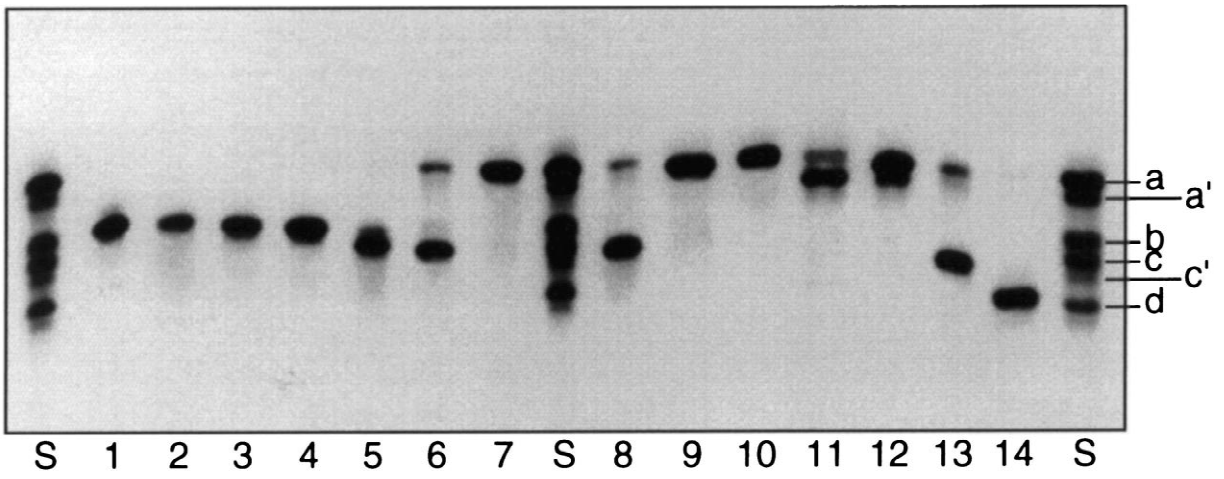

Fig. 6. AU-PAGE identification of H1 variants resolved by CX-HILIC. Pooled erythrocyte H1 was chromatographed on 100-nm pore diameter PolyCAT A as in Fig. 5B. The 4+5 and 8+9 variant peaks were subsequently chromatographed on 30 nm pore PolyCAT A as in Fig. 5A. H1 variants were recovered by TCA precipitation and analysed by AU-PAGE and silver staining. Gel lane numbering corresponds to the peak numbers used in Fig. 5. S is the pooled erythrocyte H1 starting material. The six bands resolved by AU-PAGE for the starting material are labeled according to the nomenclature used previously to identify the six known chicken H1 gene products [40].

Table 1
Assumed properties of chicken erythrocyte H1 variants based on their gene sequences

HILIC-CXC peak[a]	AU-PAGE variant[b]	Predicted net charge (pH 6.5)[c]	Predicted length[c]
1	b	+59	223
2	b	+59	223
3	b	+59	223
4	b	+59	223
5	c	+59	219
6	c	+59	219
7	a	+59	224
8	c	+59	219
9	a	+59	224
10	a	+59	224
11	a′	+58	218
12	a	+59	224
13	c′	+59	218
14	d	+60	217

[a] Peaks are numbered according to elution order in Fig. 5.
[b] Nomenclature based on mobility in AU-PAGE [40] as shown in Fig. 6.
[c] Derived from the gene sequences [39] assuming free amino- and carboxyl-termini and assigning +1 for all lysine and arginine residues and −1 for all aspartic acid and glutamic acid residues.

and H1d were also present in total H1 from pooled blood (data not shown).

3.7. Evidence for allelic polymorphism of chicken H1

The resolution of more than six H1 variants from extracts of pooled blood by CX-HILIC was unexpected and led us to examine whether post-translational modifications of H1 were involved. Glycosylation, phosphorylation and poly(ADP-ribosylation) did not appear to be involved because treatment of pooled total H1 with glycopeptidase F, alkaline phosphatase or dilute sodium hydroxide prior to chromatography did not alter the AU-PAGE and CX-HILIC profiles of total H1 from pooled blood (data not shown). None of the heterogeneity appeared to be attributable to acid extraction since the AU-PAGE and CX-HILIC profiles of total H1 prepared by salt extraction were identical to those prepared by acidic extraction (data not shown). Analyses of histone phosphorylation and acetylation have revealed that AU-PAGE can resolve modified forms differing by one unit of net charge [16,54]. Thus, the observation that the multiple forms of H1a, H1b and H1c resolved by CX-HILIC had identical mobilities in AU-PAGE confirmed that these forms were not differentially acetylated or phosphorylated. Moreover, AU-PAGE analyses appear to be sensitive to small differences in molecular size [16,19], indicating that these multiple forms were most likely to be distinguished by variations in amino acid sequence or small post-translational modifications that did not significantly affect the net charge and size of the molecules under the conditions employed for AU-PAGE.

The total H1 used to develop the CX-HILIC method was prepared from blood pooled from many chickens, so we examined the possibility that allomorphic forms of H1 were present in this sample. Comparisons of CX-HILIC profiles of H1 from the blood of single chickens with that of the pooled sample suggested that allelic polymorphism was responsible for the multiple forms of H1a, H1b and H1c in the pooled sample. Fig. 7 shows a typical comparison using 100 nm diameter pore PolyCAT A. AU-PAGE and rechromatography experiments (individual variants recovered from the single sample were rechromatographed after mixing with aliquots of the pooled sample) were used to confirm variant identifications. The putative allomorphs of H1a (variants 9 and 12), H1b (variants 1, 2 and 3) and H1c (variant 5) were not detected in the blood of this chicken (Fig. 7A). Excluding late-eluting minor species that were not characterized, the H1 set of this bird consisted of two forms of H1a (variants 7 and 10), one form of H1a′ (variant 11), one form of H1b (variant 4), two forms of H1c (variants 6 and 8), one form of H1c′ (variant 13) and one form of H1d (variant 14).

4. Discussion

4.1. Resolution of H1 variants by CX-HILIC compared to other methods

Species-specific and tissue-specific heterogeneity of H1 was first discovered using Bio-Rex 70 chromatography [15,55–57]. However, compared to the CX-HILIC method presented here, Bio-Rex 70 chromatography is time-consuming and less able to resolve

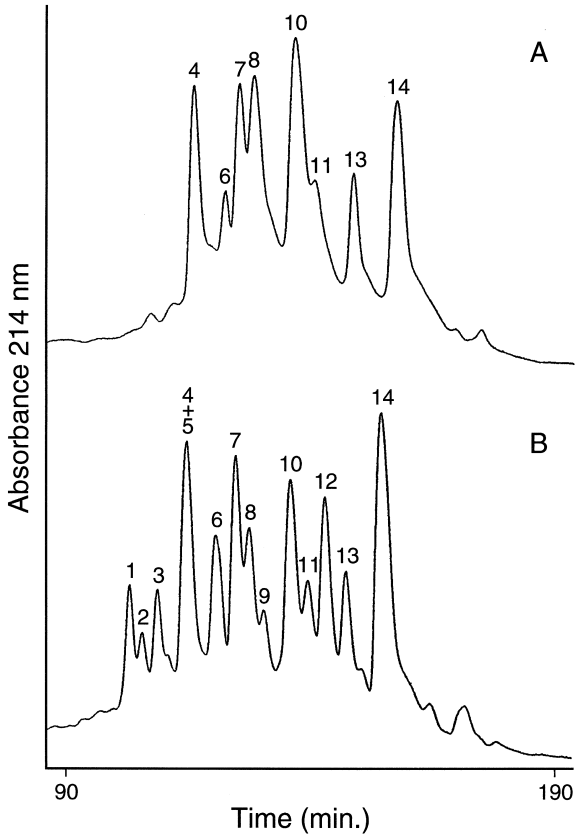

Fig. 7. Comparison of the CX-HILIC profile of erythrocyte H1 from a single chicken with that of H1 from pooled erythrocytes. Approximately 100-μg of erythrocyte H1 from (A) a single chicken and (B) 100-μg of pooled erythrocyte H1 were chromatographed on the same PolyCAT A column (100-nm diameter pores) using a 4-h linear gradient from 380 to 590 m*M* $NaClO_4$ in 10 m*M* propionic acid pH 6.5 containing 70% (v/v) acetonitrile. All other conditions were as in Fig. 1. Only the central portion of the chromatograms, aligned according to peak identification by PAGE and rechromatography of individual peaks following mixing with the heterologous sample, are shown.

chicken H1 variants. Only five components were resolved for H1 extracted from pooled chicken erythrocytes (i.e. similar to that employed here) in Bio-Rex 70 fractionations lasting 6 or 20 days [57,58]. Furthermore, guanidine hydrochloride, the gradient-forming material typically employed in histone fractionations on Bio-Rex 70, absorbs light strongly at wavelengths less than 280 nm, limiting the sensitivity of protein detection. Adaptation of the Bio-Rex 70 methodology for use with a Mono S column permitted four components of chicken erythrocyte H1 to be resolved within 30 min [40]. The CX-HILIC method described here separated all six of the chicken H1 variants described previously and resolved what appear to be allelic forms of at least three variants. In total, 14 major and several minor components were resolved during a 4-h fractionation of pooled erythrocyte H1. The buffers are compatible with UV detection at 214 nm, and we routinely obtained chromatograms with 14 major peaks from as little as 20 μg of pooled total erythrocyte H1 injected on an analytical (4.6 mm I.D.×200 mm) column. This sensitivity can be enhanced by using smaller format columns (i.e. 2.1 mm I.D.×100 mm) with only a slight loss in resolution (data not shown).

Comparison of published reports suggests that the efficacy of RP-HPLC for resolving H1 variants may be species-dependent. Mouse [26,29,34–36], rat [25,31–33] and human H1 [30] have been resolved into four or more components (excluding H1°) by different RP-HPLC methods. However, calf thymus H1 manifesting six components in Bio-Rex 70 chromatography [12,59], was resolved into only two [27,28] or three [24] components by RP-HPLC. Similarly, and in agreement with the data shown here (Fig. 1), chicken H1 eluted as a single peak in an optimized RP-HPLC method that resolved two forms of the related linker histone H5 [23]. Taken together, these observations suggest that RP-HPLC is unable to resolve H1 variants of some species, possibly those in which amino acid sequences are most highly conserved between variants. CX-HILIC clearly provides greater resolution of chicken H1 than RP-HPLC. We have found that with appropriate adjustment of the acetonitrile and sodium perchlorate concentrations employed, the method resolves greater numbers of H1 variants than RP-HPLC for human, mouse and rat samples (data not shown).

Recently, Lindner and colleagues have demonstrated the efficacy of RP-HPLC in combination with a HILIC-based method to separate acetylated forms of histones H2A and H4 [47], and phosphorylated forms of H1 [38]. Using RP-HPLC, these authors resolved two fractions for H1 from cultured human cells, one that appeared to be a single variant and a second containing several variants. Subsequent analysis of the first fraction by HILIC on a PolyCAT A column at pH 3.0 resolved several phosphorylated

forms of the purified H1 variant [38]. Ionization of the PolyCAT A support is suppressed under these conditions, suggesting that ionic interactions do not contribute to the separation of phosphorylated forms of H1 in this case. We have found that the resolution of H1 variants from chicken, human, mouse and other species by CX-HILIC on PolyCAT A is greatest when performed at pH 6.5 as described here and surpasses that achieved by RP-HPLC. It would be of interest to investigate whether CX-HILIC alone, or in combination with the HILIC method described by Lindner and colleagues, is capable of resolving complete sets of H1 variants and their phosphorylated forms.

4.2. CX-HILIC resolves allelic variants of H1

The presence of variants, not detected in CX-HILIC profiles of H1 from individual birds, in the CX-HILIC profile of H1 from the pooled blood sample (Fig. 7), strongly suggested that allelic variants of H1a, b and c were present in the pooled sample. Allelic variation in a histone was first demonstrated with the partial resolution on Bio-Rex 70 of two chicken H5 variants differing by a single amino acid substitution (Gln to Arg) [60], and it has been suggested that polymorphisms encountered during the sequencing of Bio-Rex 70 fractions of calf and rabbit H1 were due to allelic variation [12]. Systematic analyses of allelic variation in linker histones have been described in salmon [61], rabbits [62], *Xenopus* [63] mice [64] and several avian species [65–70]. In these reports, allomorphs were detected according to altered mobilities in SDS-PAGE or AU-PAGE, so only allomorphs sufficiently different in size and/or net charge were resolved. Thus, the putative allomorphs resolved by CX-HILIC (Figs. 5 and 6) represent a class of H1 variants which has not been described previously. The number and apparent abundance of these putative allomorphs suggest that allelic variants of H1 which are not resolved by electrophoretic methods are common in chickens. The detection, by mass spectroscopy, of microheterogeneity in electrophoretically homogenous fractions of mouse and bovine H1 prepared by RP-HPLC [36,71], is consistent with the occurrence of similar H1 sequence variation in these species.

4.3. Factors in the separation of H1 variants by CH-HILIC

The six known gene-derived chicken H1 protein sequences are shown aligned for maximum homology in Fig. 8. The sequences of these proteins are highly conserved and virtually all of the sequence variation occurs in the amino- and carboxyl-terminal domains. Non-conservative changes in ionizable residues are found at only five places in the entire sequence collection, corresponding to residues 10, 15, 119, 183 and 224 of the H1a sequence. Consequently, at the nearly neutral pH usually employed for cation-exchange chromatography of H1 (e.g. pH 6.5 in this report), these six proteins comprise only three values of predicted net charge, +58, +59 and +60 (Table 1). Although H1a, H1b, H1c and H1c′ differ slightly in size (224, 223, 219 and 218 residues, respectively, Table 1), they are predicted to have identical net charge at pH 6.5 (+59, Table 1). Because the positions of almost all of the ionizable residues are equivalent in these proteins (Fig. 8), and because the overall order of elution of the H1 variants in CX-HILIC does not correlate with their predicted net charge (Table 1), it seems unlikely that these four proteins were resolved on the basis of electrostatic interactions alone. Similar considerations apply to the separation of the putative allomorphs of H1a, H1b and H1c, suggesting that the resolution of both the non-allelic and the putative allelic variants of chicken H1 on PolyCAT A (Figs. 5 and 7) involved a complex mechanism.

We suggest that both electrostatic and hydrophilic interactions contribute to the resolution of H1 variants on PolyCAT A. As shown in Fig. 2, increasing levels of acetonitrile resulted in a biphasic curve for H1 retention. The reduction in retention between 0 and 40% (v/v) acetonitrile is probably not due solely to suppression of hydrophobic interactions, since less than 15% (v/v) acetonitrile was required to suppress such interactions between peptides and a similar stationary phase [72]. An increase in α-helical structure may also have contributed to the decreased retention of chicken H1 at 40% (v/v) acetonitrile but we have not confirmed this experimentally. Chicken H1 possessed considerable α-helical content under CX-HILIC conditions (e.g. 70% CH_3CN+0.6 *M* $NaClO_4$, Fig. 3), consistent with reports that TFE or

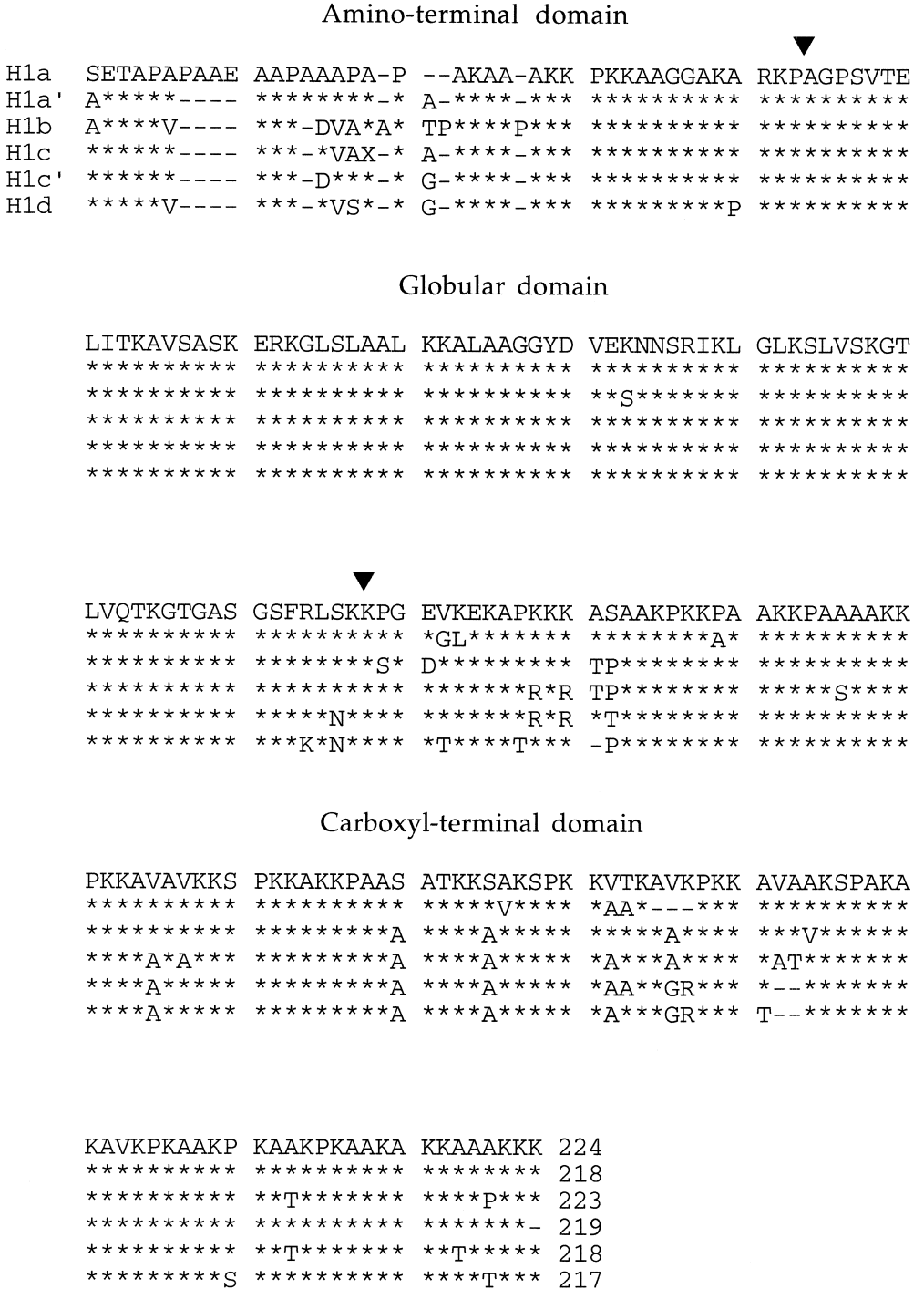

Fig. 8. Chicken H1 gene-derived protein sequences. The predicted protein sequences reported previously [39] are shown aligned for maximum homology. Stars indicate residues identical to the H1a sequence, gaps introduced for alignment are indicated by hyphens. The predicted protein lengths are indicated at the end of each sequence. The approximate boundaries of the conserved globular domain are indicated by the filled arrowheads.

$NaClO_4$ promote folding of the basic carboxyl terminal domain of H1 [53]. Even though retention of such an ordered structure by electrostatic interactions is expected to decrease because some of the basic residues formerly accessible to the stationary phase would be oriented away from it, these effects appear to have been overshadowed by increases in hydrophilic interaction since H1 retention was greater in 70% (v/v) CH_3CN than in buffer containing less CH_3CN (Fig. 2).

4.4. Application of HILIC methodologies to other proteins

Organic solvent concentrations in excess of 50% (v/v) are typically required for the retention of peptides and proteins via hydrophilic interactions on PolyCAT A and related supports [43,45,46]. Although limited solubility in common chromatographic solvents may preclude the use of HILIC-based methods for some proteins, selection of the most appropriate solvent(s) and pH may alleviate this constraint in some instances. We found that methanol promoted retention of chicken H1 by hydrophilic interactions and enabled separations of variants similar to those obtained with acetonitrile (data not shown). The efficacy of buffers containing 1-propanol for the recovery of diverse proteins from SDS-PAGE electroeluates by HILIC has been described [46]. Strong protein solvents such as dimethyl sulfoxide, formamide and ethylene glycol have been used at 100% strength in protein chromatography [73,74] and their use may facilitate HILIC-type chromatography of many proteins. The data presented here demonstrate that CX-HILIC is sensitive to small differences in H1 variant composition and suggest that HILIC-based methodologies may be suitable for analyses of protein polymorphism in general.

Acknowledgements

We thank Dr. Paul Fraser for assistance with CD spectroscopy. This work was financed by grants from the Ontario Mental Health Foundation and the Hunter Foundation.

References

[1] K.E. van Holde, Chromatin, Springer-Verlag, New York, 1989.
[2] M. Grunstein, Nature 389 (1997) 349.
[3] C.A. Mizzen, C.D. Allis, Cell Mol. Life Sci. 54 (1998) 6.
[4] P.J. Laybourn, J.T. Kadonaga, Science 254 (1991) 238.
[5] X. Shen, M.A. Gorovsky, Cell 86 (1996) 475.
[6] X. Shen, L. Yu, J.W. Weir, M.A. Gorovsky, Cell 82 (1995) 47.
[7] E.M. Bradbury, Bioessays 14 (1992) 9.
[8] S.Y. Roth, C.D. Allis, Trends Biochem. Sci. 17 (1992) 93.
[9] D.N. Chadee, C.D. Allis, J.A. Wright, J.R. Davie, J. Biol. Chem. 272 (1997) 8113.
[10] H.L. Lee, T.K. Archer, EMBO J. 17 (1998) 1454.
[11] R.D. Cole, Anal. Biochem. 136 (1984) 24.
[12] R.D. Cole, Int. J. Pept. Protein Res. 30 (1987) 433.
[13] I. Makalowska, E.S. Ferlanti, A.D. Baxevanis, D. Landsman, Nucl. Acids Res. 27 (1999) 323.
[14] J. Zlatanova, K. Van Holde, J. Cell Sci. 103 (1992) 889.
[15] J.M. Kinkade Jr., R.D. Cole, J. Biol. Chem. 241 (1966) 5790.
[16] R.W. Lennox, L.H. Cohen, Methods Enzymol. 170 (1989) 532.
[17] R.W. Lennox, L.H. Cohen, in: G.S. Stein, J.L. Stein, W.F. Marzluff (Eds.), Histone Genes: Structure, Organization and Regulation, Wiley, New York, 1984, p. 373.
[18] S.R. Sizemore, R.D. Cole, J. Cell Biol. 90 (1981) 415.
[19] L.R. Gurley, J.G. Valdez, D.A. Prentice, W.D. Spall, Anal. Biochem. 129 (1983) 132.
[20] L.R. Gurley, D.A. Prentice, J.G. Valdez, W.D. Spall, J. Chromatogr. 266 (1983) 609.
[21] L.R. Gurley, J.A. D'Anna, M. Blumenfeld, J.G. Valdez, R.J. Sebring, P.R. Donahue, D.A. Prentice, W.D. Spall, J. Chromatogr. 297 (1984) 147.
[22] P.C. Hallenbeck, R.D. Mueller, Anal. Biochem. 138 (1984) 189.
[23] W. Helliger, H. Lindner, S. Hauptlorenz, B. Puschendorf, Biochem. J. 255 (1988) 23.
[24] U. Certa, G. von Ehrenstein, Anal. Biochem. 118 (1981) 147.
[25] M. Kurokawa, M.C. MacLeod, Anal. Biochem. 144 (1985) 47.
[26] T. Wurtz, Eur. J. Biochem. 152 (1985) 173.
[27] H. Lindner, W. Helliger, B. Puschendorf, J. Chromatogr. 357 (1986) 301.
[28] H. Lindner, W. Helliger, B. Puschendorf, Anal. Biochem. 158 (1986) 424.
[29] H. Lindner, W. Helliger, B. Puschendorf, J. Chromatogr. 450 (1988) 309.
[30] Y. Ohe, H. Hayashi, K. Iwai, J. Biochem. (Tokyo) 100 (1986) 359.
[31] I. Karhu, A. Mahonen, J. Palvimo, J. Chromatogr. 426 (1988) 65.
[32] J.C. Tchouatcha-Tchouassom, J.H. Julliard, B. Roux, Biochim. Biophys. Acta 1009 (1989) 121.

[33] H. Lindner, W. Helliger, B. Puschendorf, Biochem. J. 269 (1990) 359.
[34] W. Helliger, H. Lindner, O. Grübl-Knosp, B. Puschendorf, Biochem. J. 288 (1992) 747.
[35] H. Lindner, W. Helliger, A. Dirschlmayer, H. Talasz, M. Wurm, B. Sarg, M. Jaquemar, B. Puschendorf, J. Chromatogr. 608 (1992) 211.
[36] V. Giancotti, A. Bandiera, L. Ciani, D. Santoro, C. Crane-Robinson, G.H. Goodwin, M. Boiocchi, R. Dolcetti, B. Casetta, Eur. J. Biochem. 213 (1993) 825.
[37] L.R. Gurley, D.A. Prentice, J.G. Valdez, W.D. Spall, Anal. Biochem. 131 (1983) 465.
[38] H. Lindner, B. Sarg, W. Helliger, J. Chromatogr. A 782 (1997) 55.
[39] L.S. Coles, A.J. Robins, L.K. Madley, J.R. Wells, J. Biol. Chem. 262 (1987) 9656.
[40] M.F. Shannon, J.R. Wells, J. Biol. Chem. 262 (1987) 9664.
[41] C.A. Mizzen, D.R. McLachlan, in: Fourteenth International Congress of Biochemistry, Prague, 1988, p. FR193, Abstract.
[42] C.A. Mizzen, D.R. McLachlan, T.P.A. Kruck, in: Ninth International Symposium on the HPLC of Peptides, Proteins and Polynucleotides, Philadelphia, 1989, p. 320, Abstract.
[43] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[44] B.Y. Zhu, C.T. Mant, R.S. Hodges, J. Chromatogr. 548 (1991) 13.
[45] B.Y. Zhu, C.T. Mant, R.S. Hodges, J. Chromatogr. 594 (1992) 75.
[46] P. Jenö, P.E. Scherer, U. Manning-Krieg, M. Horst, Anal. Biochem. 215 (1993) 292.
[47] H. Lindner, B. Sarg, C. Meraner, W. Helliger, J. Chromatogr. A 743 (1996) 137.
[48] A.L. Olins, R.D. Carlson, E.B. Wright, D.E. Olins, Nucleic Acids Res. 3 (1976) 3271.
[49] N. Harborne, J. Allan, FEBS Lett. 194 (1986) 267.
[50] U.K. Laemmli, Nature 227 (1970) 680.
[51] J.H. Morrissey, Anal. Biochem. 117 (1981) 307.
[52] D.J. Clark, C.S. Hill, S.R. Martin, J.O. Thomas, EMBO J. 7 (1988) 69.
[53] C.S. Hill, S.R. Martin, J.O. Thomas, EMBO J. 8 (1989) 2591.
[54] H. Talasz, W. Helliger, B. Puschendorf, H. Lindner, Biochemistry 35 (1996) 1761.
[55] J.M. Luck, P.S. Rasmussen, K. Satake, A.N. Tsetikov, J. Biol. Chem. 233 (1958) 1407.
[56] M. Bustin, R.D. Cole, J. Biol. Chem. 243 (1968) 4500.
[57] J.M. Kinkade Jr., J. Biol. Chem. 244 (1969) 3375.
[58] T. Dupressoir, P. Sautière, Biochem. Biophys. Res. Commun. 122 (1984) 1136.
[59] M.J. Smerdon, I. Isenberg, Biochemistry 15 (1976) 4233.
[60] P.J. Greenaway, K. Murray, Nature New Biol. 229 (1971) 233.
[61] A.O. Zalenskii, L.V. Brykova, F.A. Gorel', V.A. Berdnikov, I.A. Zalenskaia, Biokhimiia 46 (1981) 481.
[62] J. Palyga, Int. J. Biochem. 22 (1990) 1351.
[63] E. Dworkin-Rastl, H. Kandolf, R.C. Smith, Dev. Biol. 161 (1994) 425.
[64] A. Zweidler, in: G.S. Stein, J.L. Stein, W.F. Marzluff (Eds.), Histone Genes: Structure, Organization and Regulation, Wiley, New York, 1984, p. 339.
[65] F.L. Gorel', V.A. Berdnikov, S.M. Rozov, Mol. Biol. (Mosk) 16 (1982) 790.
[66] J. Palyga, Biochem. Genet. 28 (1990) 359.
[67] J. Palyga, Biochem. Genet. 29 (1991) 431.
[68] J. Palyga, Hereditas 114 (1991) 85.
[69] J. Palyga, Int. J. Biochem. 23 (1991) 845.
[70] J. Palyga, E. Górnicka-Michalska, A. Kowalski, Biochem. J. 294 (1993) 859.
[71] R.G. Berger, R. Hoffmann, M. Zeppezauer, W. Wagner-Redeker, L. Maljers, A. Ingendoh, F. Hillenkamp, J. Chromatogr. A 711 (1995) 159.
[72] B.-Y. Zhu, C.T. Mant, R.S. Hodges, J. Chromatogr. 594 (1992) 75.
[73] N. Chang, S.J. Hen, A.M. Klibanov, Biochem. Biophys. Res. Commun. 176 (1991) 1462.
[74] N. Chang, A.M. Klibanov, Biotech. Bioeng. 39 (1992) 575.