Linker Histone Subtype Composition and Affinity for Chromatin in Situ in Nucleated Mature Erythrocytes*

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Elisavet Koutzamani‡‡, Helena Loborg‡‡, Bettina Sarg‡, Herbert H. Lindner‡, and Ingerman Rundquist‡‡

From the ‡Department of Biomedicine and Surgery, Division of Cell Biology, Faculty of Health Sciences, Linköpings universitet, SE-581 83 Linköping, Sweden and the Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Fritz Preglstrasse 3, A-6020 Innsbruck, Austria

The replacement linker histones H10 and H5 are present in frog and chicken erythrocytes, respectively, and their accumulation coincides with cessation of proliferation and compaction of chromatin. These cells have been analyzed for the affinity of linker histones for chromatin with cytotechnical and biochemical methods. Our results show a stronger association between linker histones and chromatin in chicken erythrocyte nuclei than in frog erythrocyte nuclei. Analyses of linker histones from chicken erythrocytes using capillary electrophoresis showed H5 to be the subtype strongest associated with chromatin. The corresponding analyses of frog erythrocyte linker histones using reverse-phase high performance liquid chromatography showed that H10 dissociated from chromatin at somewhat higher ionic strength than the three additional subtypes present in frog blood but at lower ionic strength than chicken H5. Which of the two H1 variants in frog is expressed in erythrocytes has thus far been unknown. Amino acid sequencing showed that H10-2 is the only H1 subtype present in frog erythrocytes and that it is 100% acetylated at its N termini. In conclusion, our results show differences between frog and chicken linker histone affinity for chromatin probably caused by the specific subtype composition present in each cell type. Our data also indicate a lack of correlation between linker histone affinity and chromatin condensation.

Eukaryotic chromatin is a dynamic entity exhibiting different levels of compaction, and its intrinsic flexibility entails reversible switches between decondensed and condensed states for proper transcription and replication. The histone proteins, the core histones H2A, H2B, H3, and H4, and the linker histones, commonly referred to as H1, interact with DNA to form nucleosomes and higher order structures of the chromatin fiber (1). The flexibility of this interaction enables histone participation in the modulation of transcriptional activity. H1 was considered initially to be a structural protein increasing the compaction of chromatin and, in addition, mediating global silencing of gene activity (2). Although its physiological function is still an enigma, evidence has been put forward emphasizing a gene-specific regulation, mostly involving gene silencing (3).

The H1 isoforms have in common a tripartite protein structure. The globular domain is the most conserved part whereas the N- and C-terminal tails contain the most variation in amino acid sequence and are thus responsible for the microheterogeneity of H1 histones (4). The tails are targets for several post-translational modifications of which phosphorylation of serine and threonine residues has been the most studied. Phosphorylation has been linked to both condensation and decondensation of chromatin (5), and recent results indicate that phosphorylation of linker histones regulates gene expression in vivo by causing their partial dissociation from chromatin (6). Furthermore, linker histone phosphorylation and partial dissociation may be a first step in the regulation of ATP-dependent chromatin remodeling enzymes (7).

The number of linker histone subtypes and their amino acid composition varies among species, as well as tissues (8). The isoforms differ in the timing of expression during development, in the number of phosphate groups they can accept during cell division (4), and in their ability to condense chromatin (9, 10). Their association to chromatin has been shown to be selective, forming stronger bonds upon association with AT-rich sequences (8, 11, 12). Although it appears as if each of the linker histone subtypes may have a distinct function, elimination of certain subtypes by knockout experiments shows that they are dispensable and that the remaining subtypes can compensate for the lost ones (13, 14).

A specific subtype, H10, has been shown to accumulate in many terminally differentiated cells, and it has been suggested that H10 may contribute to cell differentiation (15). In the nucleated erythrocytes present in avian and amphibian organisms the replacement linker histones, H5 and H10, respectively, play a key role in structuring the highly condensed and inert chromatin that is representative of this type of differentiated cells. In Xenopus laevis erythrocytes, a 3-fold increase in the relative content of H10 was demonstrated during erythroid maturation (16).

The binding of H1 to chromatin is mediated mainly by ionic interactions, and it is weaker than that of core histones. A well established and gentle method to follow the dissociation of H1 from chromatin is the selective extraction of H1 using salt concentrations in the range of 0.35 to 0.7 M (17, 18). We have combined this dissociation of H1 with the use of the DNA-binding fluorochrome DAPI to establish an indirect cytochemical method to measure the affinity of linker histones for chromatin in situ (19, 20).

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‡ Contributed equally to this work.
§ To whom correspondence should be addressed. Tel.: 46-13-224395; Fax: 46-13-224314; E-mail: ingru@mch.liu.se.

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In the present work we have applied this cytofluorometric method to define changes in chromatin structure between mature chicken and frog erythrocytes containing different subsets and amounts of H5 and H1. To verify the dissociation of linker histones at the protein level, samples isolated at different salt concentrations were analyzed by electrophoretic and chromatographic methods. Our results show a clear difference in the affinity of linker histones to chromatin between frog and chicken erythrocytes. These results implicate that the condensation of chromatin is not mediated solely by linker histones. Other key factors interacting with chromatin may be necessary to produce the inert state of highly condensed chromatin.

EXPERIMENTAL PROCEDURES

Chemicals

DAPI, digitonin, 3-aminobenzoic acid ethyl ester (MS-222), protamine sulfate, succrose, N\textsuperscript{2}-p-tosyl-L-lysine chloromethyl ketone (TLCK), and Trizma (Tris base) were purchased from Sigma. Acetoneitrile, apro- tinin, E-64, hydroxypropylmethyl cellulose (HPMC, 4000 centipoise) and trichloroacetic acid were purchased from Fluka (Buchs, Switzerland). Bestatin, leupeptin, and pepstatin were purchased from Becham (Switzerland), ethylene glycolmonomethylether (EGME) was from Aldrich (Steinheim, Germany), and sequencing grade chymotrypsin was from Roche Molecular Biochemicals. All other chemicals were purchased from Merck (Darmstadt, Germany).

Collection of Blood and Tissues

Blood from chicken was collected into heparinized tubes. For cytofluorometric analyses, blood from adult female X. laevis was obtained by cutting a vein of the web and collected in heparinized capillary tubes. For linker histone analyses frog blood was obtained from animals anesthetized with 0.25% MS-222 by heart puncture and collected into hepar- inized tubes. Frog liver was removed immediately after heart puncture and rinsed with frog PBS to remove blood.

Cell Preparations for Cytochemistry

Unextracted (Intact) Cells—The cells were fixed in 4% neutral buffered paraformaldehyde (PFA) for 30 min (0.5–2 × 10\(^6\) cells/ml), and aliquots (150–175 \(\mu\)l) of the cell suspension were placed on cover glasses pretreated with 0.005% protamine sulfate. The cells were allowed to adhere to the glasses for 10 min on ice and were then kept in 4% PFA for 2–5 days. Immediately before staining the cells were washed in a Tris-NaCl buffer (10 mM Tris-HCl, 50 mM MgCl\(_2\), and 150 mM NaCl, pH 7.4, for 30 min, permeabilized with 40 \(\mu\)g/ml digitonin in Tris-NaCl buffer for 10 min, and washed again in 1 min.

Salt-extracted Cells—Unfixed chicken erythrocytes were diluted in PBS (2.68 mM KCl, 1.47 mM KH\(_2\)PO\(_4\), 8.1 mM NaHPO\(_4\), and 137 mM NaCl), and unfixed frog erythrocytes were diluted in frog PBS (1.79 mM KCl, 0.985 mM KH\(_2\)PO\(_4\), 5.4 mM NaHPO\(_4\), and 91.3 mM NaCl). Cells in suspension (0.5–2 × 10\(^6\) cells/ml) were allowed to adhere to cover glasses as indicated above and were subsequently permeabilized with 40 \(\mu\)g/ml digitonin in Tris-NaCl buffer for 10 min. The cells were either fixed directly in 4% PFA for 2–5 days or extracted with different concentrations (0.15 to 0.75 mM) of NaCl in Tris-NaCl buffer for 5 min and subsequently fixed in 4% PFA for 2–5 days. The salt extraction buffer was supplemented with 1 mM succrose to prevent disruption of the cells. All preparations were performed on ice. Before staining, the cells were washed with Tris-NaCl for 30 min at room temperature.

Cell Staining

A 0.5 mM stock solution of DAPI was prepared by dissolving the dye in sterile Millipore water. The solution was stored at 4 °C and used within one month. The cells were stained with 50 nM DAPI in Tris-NaCl buffer at room temperature until equilibrium was reached (i.e. at least 1.5 h). The volume of the staining solution was large enough to maintain a constant concentration of the stain. Immediately before cytofluorometry the cover glasses were mounted on a slide with printed rings (Erie Scientific, Portsmouth, NH) that constituted thin chambers filled with DAPI stain.

Cytofluorometry

The fluorescence intensity (FI) of the cells was measured by image cytofluorometry, using a Nikon Microphot FXA fluorescence microscope equipped with an Ultrapan 2000 cooled CCD camera (EG & G WALLAC LSR, Cambridge, United Kingdom). The microscope was equipped with a stabilized xenon arc lamp (Oriel XBO 100 W) for incident fluorescence excitation. A standard filter set for DAPI (Nikon UV-2A) was employed that included a 330–380-nm excitation filter, a dichromatic beam splitter with 50% reflection at 400 nm, and a 420-nm barrier filter. A Nikon CF Fluor objective (10×, numerical aperture = 0.5) was used for the measurements.

The integrated FI of each cell within the camera frame area (1392×1040 pixels, intensity resolution = 8 bits/pixel) was calculated using the image-processing program Image-Pro Plus 4.0 (Media Cybernetics). The camera gain parameters were set in such a way that pixel saturation was avoided and kept constant for each series of measurements. An average background fluorescence (10–12 units/pixel) was subtracted from each image, and a threshold of 5 units was then employed to delimit the fluorescent structures before the calculation of their integrated FI. The number of cells per frame was about 50 and their mean integrated fluorescence was used for the calculations of H1 affinity.

Four frames on each cover glass were analyzed, and each experiment included data from duplicate glasses, which means that about 400 cells were measured for each data point within a series of measurements.

The analysis of salt extraction curves was performed as described previously (19, 20) using a least-squares curve fitting to a linker histone binding equation (18). The salt extraction curves were normalized using the FI after extraction with 0.15 mM NaCl as 100%. The NaCl concentra- tion required to induce a 50% increase in FI was then calculated from the fitted equation and used as a measure of an apparent average linker histone affinity for chromatin in situ. The statistical significance of differences between results was analyzed using unpaired Student’s t test. Images to be used as fluorescence micrographs were obtained from the CCD camera using a Nikon CF Fluor objective (40×, numerical aperture = 0.85).

Protease Inhibitors

The following protease inhibitors were added to all buffers used for the isolation of H1 from frog blood: aprotinin (2 \(\mu\)g/ml), bestatin (40 \(\mu\)g/ml), E-64 (2 \(\mu\)g/ml), leupeptin (5 \(\mu\)g/ml), pepstatin (5 \(\mu\)g/ml), phenylmethylsulfonyl fluoride (2 mM), and TLCK (50 \(\mu\)g/ml). For isolation of H1 from chicken erythrocytes only phenylmethylsulfonyl fluoride (1 mM) was used.

Isolation of H1 with Perchloric Acid for H1 Subtype Analysis

After collection, frog blood was washed three times (500 × g, 10 min, 4 °C) in frog PBS with the addition of 10 mM 2-mercaptoethanol. Chicken blood was washed three times in Tris-HCl NaCl buffer with the addition of 10 mM 2-mercaptoethanol. After washing, the cells were permeabilized in STKM buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl\(_2\), and 10 mM 2-mercaptoethanol) including 0.2% Triton X-100. Nuclei were prepared by using 10 strokes for chicken erythrocytes and five strokes for frog erythrocytes in a Dounce homogenizer. The nuclei were centrifuged at 1500 × g for 10 min at 4 °C and subsequently washed once in STKM buffer without detergent.

Frog liver was homogenized in 2 volumes of STKM buffer and filtrated through several layers of gauze and centrifuged at 2000 × g for 10 min at 4 °C. The pellet was washed three times in STKM buffer and then homogenized with STKM buffer containing 0.2% Triton X-100 in a Dounce homogenizer.

H1 was extracted from blood and liver by the addition of one volume 10% perchloric acid (PCA) and 4 volumes of 5% PCA to the nuclear suspension, and the mixture was incubated for 1 h on ice. Insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4 °C. H1 was recovered from the supernatant by adding 100% trichlo- roacetic acid to a final concentration of 20%. To allow precipitation, the samples were left on ice for 1 h. The sample was centrifuged at 12,000 × g for 10 min at 4 °C, and the pellet was washed once in cold acidified acetone and then once with pure acetone (12,000 × g, 10 min, 4 °C). The precipitated sample was dissolved in water with 10 mM 2-mercaptoethanol and lyophilized.

Stepwise Extraction of H1 with Sodium Chloride for H1 Subtype Analysis

To dissociate linker histones with different concentrations of sodium chloride, the erythrocytes were permeabilized with 40 \(\mu\)g/ml digitonin in Tris-NaCl buffer with the addition of 10 mM 2-mercaptoethanol for 10 min on ice, and centrifuged at 1500 × g for 10 min at 4 °C. The permeabilized cells were treated with different concentrations of sodium chlor- ide (0.35, 0.4, 0.45, 0.5, or 0.7 mM) with the addition of 10 mM 2-mercap- toethanol for 5 min on ice. Insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4 °C. To the supernatant, 40%
trichloroacetic acid was added to a final concentration of 2% and left on ice for 1 h. Insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4 °C, and 100% trichloroacetic acid was added to the supernatant to a final concentration of 20% in the presence of 10 mg/ml protamine sulfate. The sample was incubated for 1 h on ice and then centrifuged at 12,000 × g for 10 min at 4 °C. The pellet was washed with acidified and pure acetone, dissolved in water with 10 mM mg/ml protamine sulfate. The sample was incubated for 1 h on ice and then centrifuged at 12,000 × g for 10 min at 4 °C. The pellet was washed with acidified and pure acetone, dissolved in water with 10 mM 2-mercaptoethanol, and lyophilized as described above.

Capillary Electrophoresis
High performance capillary electrophoresis (HPCE) was performed on a Beckman system PE/ACE 2100 controlled by an AT486 computer. Data collection and post-run data analyses were carried out using PE/APE and System Gold software (Beckman Instruments). The capillary cartridge used was fitted with 75-μm internal diameter fused silica of 67 cm total length (60 cm to the detector). In all experiments an untreated capillary was used. Protein samples were injected by pressure, and detection was performed by measuring UV absorption at 200 nm. Separation of H1 was performed as described (21–24). Linker histones from chicken erythrocytes were analyzed in 0.1 M phosphate buffer containing 180 mM HCIO4 and 0.02% HPMC, and linker histones from frog erythrocytes and frog liver were analyzed in 0.1 M phosphate buffer containing 0.02% HPMC. All runs were carried out at a constant voltage (12 kV) and at a capillary temperature of 25 °C.

Reverse-phase high performance liquid chromatography (RP-HPLC)
A 127 Solvent Module and a Model 168 UV-visible-region detector (Beckman Instruments, Palo Alto, CA) were used. The effluent was monitored at 210 nm, and the peaks were recorded using Beckman System Gold software.

The separation of frog linker histones was performed on a Nucleosil 300–5 C4 column (8.0-mm internal diameter × 125 mm; 5-μm particle pore size; 30-nm pore size; end-capped; Macherey-Nagel, Düren, Germany). The lyophilized proteins were dissolved in water containing 0.2 M 2-mercaptoethanol and injected onto the column. Separation of H1 was performed as described (25–27). Briefly, linker histones from frog erythrocytes were chromatographed at a constant flow of 1.0 ml/min with a acetonitrile gradient starting at solvent A:solvent B ratio of 90:10 (solvent A, water containing 0.1% trifluoroacetic acid; solvent B, 85% acetonitrile and 0.1% trifluoroacetic acid). The concentration of solvent B was increased from 20 to 50% (v/v) in 50 min and from 50 to 100% (v/v) in 15 min. Linker histones from frog liver were fractionated as described for erythrocytes. The histone fractions were collected, and after the addition of 10 mM 2-mercaptoethanol the sample was lyophilized and stored at −20 °C.

Chymotrypsin Digestion and Amino Acid Sequence Analysis
H1(−4 μg) obtained from frog erythrocytes by RP-HPLC fractionation (see Fig. 4) was digested with α-chymotrypsin (EC 3.4.21.1) (Sigma type I-S; 1:150 (w/w)) in 20 μl of 100 mM sodium acetate buffer (pH 5.0) for 2 h at room temperature. The peptides obtained were separated using a Nucleosil 300–5 C18 column (150 × 2-mm inner diameter; 5-μm particle pore size; end-capped; Macherey-Nagel, Düren, Germany). Samples of −4 μg were injected onto the column. Chromatography was performed within 70 min at a constant flow of 75 μl/min with a two-step acetonitrile gradient starting at solvent A:solvent B ratio of 90:10 (solvent A, water containing 0.1% trifluoroacetic acid; solvent B, 85% acetonitrile and 0.1% trifluoroacetic acid). The concentration of solvent B was increased linearly from 10 to 45% during 65 min and from 45 to 100% during 10 min. Fractions obtained in this way were collected and, after adding 20 μl of 2-mercaptoethanol (0.2 M), lyophilized. Corresponding peptide fractions were redissolved in 20 μl of 0.1% trifluoroacetic acid and spotted onto a polyvinylidene difluoride membrane. After washing the membrane twice with 20 μl of 0.1% trifluoroacetic acid the adsorbed peptide was sequenced using an Applied Biosystems Inc. (ABI) Model 492 Procise protein sequenator.

RESULTS

Cytofluorometry—Fluorescence was confined to nuclear chromatin, and background fluorescence was low in relation to nuclear fluorescence intensity. Although extraction with sodium chloride made the cells more fragile, careful handling during the preparation steps resulted in cells that were well suited to be measured by image cytofluorometry. Nuclei in intact erythrocytes from adult animals of both avian and amphibian origin were small and showed distinct heterogeneous DAPI staining. Permeabilization using digitonin in adequate concentration is supposed to be a gentle method, only affecting the plasma membrane (28). Phase contrast microscopy showed that the erythrocytes appeared to be relatively undamaged, independently of whether they were permeabilized before or after fixation. However, for both cell types permeabilization with digitonin increased the fluorescence intensity about 2-fold and resulted in a slightly larger nucleus with a more homogeneous staining pattern. Treatment with 0.15 M NaCl after permeabilization did not result in any change in the nuclear appearance or a further increase of erythrocyte fluorescence intensity in accordance with our previous results (20).

The salt concentration needed to induce a half-maximal increase in FI in chicken erythrocytes (Fig. 1A) was 0.55 ± 0.02 M NaCl (n = 5), which was higher than the concentrations needed for a similar increase in other cell types studied (20). The salt concentration needed to induce the corresponding increase in FI in frog erythrocytes (Fig. 1B) was significantly lower, 0.374 ± 0.003 M NaCl (n = 5, p < 0.001) (Fig. 1B), than in chicken erythrocytes (Fig. 1A). Chicken erythrocytes extracted by 0.4 M NaCl (Fig. 2B) showed an appearance similar to unextracted cells (Fig. 2A) when examined by fluorescence microscopy, in accordance with the cytofluorometry data (Fig. 1A). At 0.7 M NaCl DAPI-stained chromatin could be observed in the cytoplasm (Fig. 2C). Frog erythrocytes showed increased FI from about 0.3 M NaCl, and no further increase was detectable after 0.55 M NaCl (Fig. 1B), in accordance with our previous results (20). Also, in accordance with the corresponding cytofluorometry data (Fig. 1B), these cells showed leakage of DAPI-stained chromatin in the cytoplasm already at 0.4 M NaCl (Fig. 2B). The total increase in FI was somewhat higher for chicken (about 30%) than frog (about 25%) erythrocytes (Fig. 1).

HPCE Separation of Linker Histone Subtypes from Chicken Erythrocytes—For the simultaneous separation of all six H1

FIG. 1. Linker histone dissociation curves derived from the average relative fluorescence intensity (n = 5) as a function of NaCl concentration. The curves represent chicken erythrocytes (A) and frog erythrocytes (B). Fluorescence intensity values were obtained after staining with 50 nM DAPI. The bar through each data point indicates its standard error of the mean.
variants and the replacement histone H5 present in chicken erythrocytes, separation conditions first described by Lindner and co-workers (24, 29) were applied. Excellent separation was obtained by using a buffer containing 0.02% of the coating agent HPMC, 100 mM H₃PO₄, and 180 mM HClO₄. In the electropherogram obtained by analyzing PCA-extracted linker histones from chicken erythrocytes, the faster migrating peaks correspond to H5 non-acetylated and acetylated variants and the slower migrating six peaks correspond to the six H1 variants (Fig 3A). H5 represented 59 ± 8% (n = 3) of the total amount of linker histones.

The dissociation of the linker histone subtypes from chicken erythrocytes was also monitored by capillary electrophoresis after extraction with 0.7 M NaCl (Fig. 3B) and 0.5 M sodium chloride (Fig. 3C). As expected, H5 exhibited a stronger association with chromatin. It dissociated mainly at sodium chloride concentrations higher than 0.5 M, and at 0.7 M NaCl H5 represented 51 ± 5% (n = 3) of the total amount of linker histones. Only small amounts of H5 (7 ± 6%, n = 3) were detected at 0.5 M NaCl.

Isolation and Separation of H1 from X. laevis Erythrocytes—Analyses of frog H1 revealed some unexpected obstacles. Frog linker histones were isolated initially by applying the same PCA extraction procedure used for the chicken erythrocyte linker histones. The material obtained was, however, not well separated when analyzed with HPCE (data not shown), probably as a result of proteolysis. Phenylmethylsulfonyl fluoride as the sole protease inhibitor was not efficient enough to repress the very aggressive proteolytic enzymes present in Xenopus (30). The combination of the protease inhibitors described under “Experimental Procedures” resulted in the least degradation and was added to all buffers used to isolate H1. However, even then, using the same separation conditions as for chicken linker histones, the separation was not satisfying (data not shown). Very broad and not base-line separated peaks were obtained. A variety of buffers were tested with similar results. Accordingly, we changed the separation method and used RP-HPLC for the separation of frog histones. With this method a clear separation of H10 and the three other H1 variants present in X. laevis was possible (Fig. 4A). After PCA extraction H10 represented 13.3 ± 4.4% (n = 2) of the total amount of linker histones.

Frog erythrocytes were also extracted with 0.7 M NaCl (Fig. 4B), 0.5 M NaCl (Fig. 4C), and 0.45 M NaCl (not shown). After extraction with 0.7 M NaCl H10 represented 16.6 ± 1.3% (n = 2) of the total amount of linker histones, and at 0.5 M NaCl the relative amount of H10 was 6.2 ± 2.8% (n = 2). At 0.45 M NaCl it was about 3% of the total amount of H1.

Characterization of X. laevis H10—A question that arose during the separation of frog erythrocyte linker histones concerned the origin of the H10 peak (Fig. 4). X. laevis has two genes encoding the two isoforms H10-1 and H10-2 (31), also designated H5B and H5A (32). One of these isoforms has been shown to be the predominant form in erythrocytes (33). To our knowledge, no conclusive determination has so far been made to identify the H10 in frog erythrocytes. To characterize frog erythrocyte H10 and, in addition, to investigate whether the two H10 variants can be separated with RP-HPLC we analyzed H1 from frog liver, which is known to contain both H10 isoforms in nearly equal amounts (33). The two H10 isoforms were separated successfully (Fig. 5), and fractions from the two peaks were collected and digested with chymotrypsin. The resulting peptide fragments were separated and isolated by RP-HPLC (data not shown), and the corresponding fractions were subjected to amino acid sequence analysis for characterization.
Comparison with the Swiss Protein Database showed that the first peak in Fig. 5 corresponded to H10-2 and the second peak to H10-1. Fractions from the first peak in Fig. 4 were then subjected to the same analysis as the liver fractions, and the results showed that this peak contained only H10-2. The small peak between the H10 peak and the other H1 variants was identified as HMG-X. No H10-1 was detected in any fraction after separation of frog erythrocyte linker histones. Both liver and erythrocyte H10-2 were shown to be 100% acetylated at their N termini.

**DISCUSSION**

The replacement histones H5 and H10 are known to accumulate in the nuclei of terminally differentiated erythrocytes of chicken and frog, respectively. We have developed a novel cytochemical method for the in situ characterization of the affinity of linker histones for chromatin (19) and applied it to these cell types. The fluorescence measurements were performed on whole nuclei, and thus the results obtained represent an average affinity of linker histones for chromatin within individual nuclei for a small population of cells. In both cell types the replacement linker histones are major constituents of the nuclei and have been ascribed similar biological functions. Overexpression of each subtype resulted in similar outcome including retardation of cell cycle progression and increased resistance to micrococcal nuclease digestion (34–37). However, our cytofluorometric results show clearly that the increase in DAPI fluorescence occurred at higher salt concentrations for chicken erythrocytes as compared with frog erythrocytes, with almost no overlap between the two curves (Fig. 1). Our results therefore indicate that the association of linker histones is significantly stronger in chicken erythrocytes than in frog erythrocytes. Because the dominant linker histone subtype in mature chicken erythrocytes is H5 this interpretation is supported by previous results showing that H5 dissociated from chromatin at significantly higher salt concentrations than other linker histone subtypes present in chicken erythrocytes did (18). Although chromatin components other than linker histones may dissociate within the same salt concentration interval, and possibly also affect DAPI fluorescence, our present results indicate that the major part of the increase in DAPI fluorescence is actually derived from linker histone dissociation and thus provide a further validation of the specificity of our indirect cytofluorometric method.

Previous results suggest that trout “H5-like” histones were bound less tightly to erythrocyte chromatin as compared with chicken H5 but also that this H5-like protein was bound more strongly to chromatin than other H1 variants (38). Furthermore, recent results show that H10 in mouse 3T3 cells was released at slightly higher salt concentrations than a somatic H1 subvariant (39). In contrast, although not completely comparable, the addition of nucleoplasm to *Xenopus* erythrocytes resulted in H10 being removed more efficiently than other H1 subtypes present (40). Furthermore, H10 was less successful than H1–1 in protecting DNA reconstituted with H10 or H1–1 against digestion with micrococcal nuclease (10). Our present and previous results (20) show a sharp increase in DAPI fluo-
residence between 0.3 and 0.4 M NaCl in Xenopus erythrocytes (Fig. 1A), which suggests that these H1\(^6\)-enriched linker histones are bound less tightly to chromatin as compared with H1 in other cell types containing predominantly somatic linker histone variants (20). However, these discrepancies may have several explanations, and linker histone affinity in vivo is probably controlled at several levels beyond linker histone subtype composition. There may also be differences between species, as well as methodological differences. For example, we must consider the possibility that the increase in DAPI fluorescence following increasing salt concentrations may not require the release of the entire linker histone molecule; a partial dissociation may expose additional DAPI binding sites before the complete dissociation of the protein and its appearance in solution.

The dissociation behavior of the linker histones was also monitored by electrophoretic and chromatographic methods. Using HPCE we could resolve all subtypes within 36 min of separation (Fig. 3). From these analyses we conclude that the amount of H5 is about 60% of the total amount of linker histones in mature chicken erythrocytes and, as expected, that H5 dissociates at higher salt concentrations than other H1 subtypes. The results thus support the high linker histone affinity observed in these cells by our cytofluorometric measurements. The corresponding RP-HPLC analyses of frog linker histones showed that the fraction of H1\(^6\) in frog erythrocytes was about 16%, which was less than the fraction of H5 in chicken erythrocytes, indicating that chicken histones may have a higher linker histone content per nucleosome than frog erythrocytes. This conclusion is also supported by the cytofluorometry data, which showed a higher increase in DAPI fluorescence in chicken erythrocytes upon salt extraction (Fig. 1). Frog H1\(^6\) dissociated to a larger extent than H5 at 0.5 M salt, which support our cytofluorometric affinity data, but it is also clear from our data that the increase in FI precedes the biochemically detected linker histone dissociation, suggesting either that our cytochemical method is more sensitive or that it is able to detect a partial linker histone dissociation from chromatin.

A feature that is supposed to reduce the affinity of linker histones for chromatin by neutralizing their positive charge is the addition of phosphate groups to serine and threonine residues (41). During avian erythropoiesis, H5 becomes dephosphorylated progressively, and this event coincided with nuclear condensation (42, 43). In terminally differentiated Xenopus erythrocytes linker histone phosphorylation is also absent (44), and we could not detect any specific immunofluorescence when using an antibody against phosphorylated linker histones (data not shown), suggesting that these cells also contain dephosphorylated linker histones. Recently, Sarg et al. (29) found that H5 in mature chicken erythrocytes undergoes acetylation at the N terminus and that both unacetylated and acetylated forms are present. No other H1 subtypes in these cells are known to contain any modifications although some core histones were found to be acetylated (45). To our knowledge, the presence of eventual histone modifications in mature frog erythrocytes has not been investigated, but it seems unlikely that post-translational modifications are the reason for the large differences in linker histone affinity for chromatin found between chicken and frog erythrocytes.

Instead, the underlying differences in the interaction between specific linker histone subtypes and chromatin may be a reflection of their contents of the basic amino acids arginine and lysine (46). Avian H5 has a content of 22/44 arginine/lysine residues whereas Xenopus H1\(^6\)–2 contains 7/58 arginine/lysine residues. The relative high arginine content in H5, combined with H5 constituting a major part of the total linker histone content, may be sufficient to explain the high average linker histone affinity for chicken erythrocyte chromatin reported here.

We have also for the first time identified which H1\(^6\) variant is predominant in Xenopus erythrocytes. Although mRNA of both H1\(^6\) variants was found in Xenopus blood (31), our results show clearly that only H1\(^6\)-2 accumulated in Xenopus erythrocytes. Our result thus support the previous suggestion that one of the H1\(^6\) genes became erythrocyte-specific during the evolution of birds (31) and indicate that H1\(^6\)-2 is the most likely candidate for this event.

Although linker histones have been ascribed general chromatin fiber condensing properties, our results indicate that their affinity for chromatin is not the key factor for chromatin condensation and the presumed concomitant global gene inactivation in mature nucleated erythrocytes. Other nuclear factors, such as the myeloid and erythroid nuclear termination stage-specific protein, which was found in chicken granulocytes and erythrocytes, may be involved in chromatin condensation at a regulatory level (47, 48). In addition, Eufemi et al. (49) have detected a subset of proteins cross-linked to DNA in nuclei of mature chicken erythrocytes. To our knowledge, no specific factors besides linker histones have been found in mature frog erythrocytes that could be ascribed condensing properties. Nevertheless, it is highly probable that the mechanisms behind the functional organization of chromatin higher order structures generally involve linker histones, in combination with other nuclear factors, including the post-translational modification of core histones (50).

REFERENCES

1. Wolffe, A. P. (1998) Chromatin Structure and Function, 3rd Ed., Academic Press
2. Weintraub, H. (1984) Cell 38, 17–27
3. Wolffe, A. P., Khochbin, S., and Dimitrov, S. (1997) Bioessays 19, 249–255
4. Parvegnian, M. H., and Hamkalo, B. A. (2003) Biochem. Cell Biol. 79, 289–304
5. Roth, S. Y., and Allis, C. D. (1992) Trends Biochem. Sci. 17, 93–98
6. Duy, Y., Mizzen, C. A., Abrams, M., Allis, C. D., and Gorovoy, M. A. (1999) Mol. Cell 4, 641–647
7. Hor, P. J., Carruthers, L. M., Logie, C., Hill, D. A., Solomon, M. J., Wade, P. A., Imbalzano, A. N., Hansen, J. C., and Peterson, C. L. (2002) Nat. Struct. Biol. 9, 263–267
8. Cole, R. D. (1997) Int. J. Pept. Protein Res. 30, 433–440
9. Nagaraja, S., Delcue, G. F., and Davie, J. R. (1995) Biochim. Biophys. Acta 1260, 207–214
10. Marion, C., Roche, J., Roux, B., and Gerc, K. (1985) Biochemistry 24, 6328–6335
11. Zlatanova, J., and van Holde, K. (1992) J. Cell Sci. 103, 889–895
12. Zlatanova, J., and van Holde, K. (1996) Prog. Nucleic Acids Res. Mol. Biol. 52, 217–259
13. Khochbin, S. (2001) Gene 271, 1–12
14. Fan, Y., Sirountin, A., Russell, G. R., Ayala, J., and Skoultchi, A. I. (2001) Mol. Cell. Biol. 21, 7833–7843
15. Zlatanova, J., and Donecke, D. (1994) FASEB J. 8, 1260–1268
16. Rutledge, R. G., Neelin, J. M., and Seligy, V. L. (1994) Eur. J. Biochem. 144, 191–198
17. Boland, L. A., and Johns, E. W. (1973) Eur. J. Biochem. 35, 546–553
18. Kumar, N. M., and Walker, I. O. (1980) Nucleic Acids Res. 8, 3535–3551
19. Leborg, H., and Rundquist, I. (1997) Cytometry 29, 212–219
20. Leborg, H., and Rundquist, I. (2000) Cytometry 40, 1–9
21. Lindner, H., Wurm, M., Dirschlamer, A., Sarg, B., and Helliger, W. (1993) Electrophoresis 14, 480–485
22. Lindner, H., Helliger, W., Dirschlamer, A., Jaquemar, M., and Pursendorf, B. (1992) Biochem. J. 283, 467–471
23. Lindner, H., Helliger, W., Dirschlamer, A., Talasaz, H., Wurm, M., Sarg, B., Jaquemar, M., and Pursendorf, B. (1992) J. Chromatogr. 608, 211–216
24. Lindner, H., Helliger, W., Sarg, B., and Meraner, C. (1995) Electrophoresis 16, 604–610
25. Lindner, H., Wesierska-Gadek, J., Helliger, W., Pursendorf, B., and Sauermann, G. (1989) J. Chromatogr. 472, 243–249
26. Lindner, H., Helliger, W., and Pursendorf, B. (1988) J. Chromatogr. 450, 309–316
27. Helliger, W., Lindner, H., Hauptlorenz, S., and Pursendorf, B. (1988) Biochem. J. 255, 23–27
28. Ablan, S. A., Marr, R. S., and Gereas, L. (1990) J. Cell Biol. 111, 807–816
29. Sarg, B., Helliger, W., Heerdt, B., Pursendorf, B., and Lindner, H. (1999) Arch. Biochem. Biophys. 372, 333–339
30. Destree, O. H., D’Adelhart-Toorop, H. A., and Charles, R. (1975) Biochim. Biophys. Acta 378, 455–458
31. Brocard, M. P., Triebe, S., Peretti, M., Doenecke, D., and Khochbin, S. (1997) Gene 189, 127–134
32. Rutledge, R. G., Neelin, J. M., and Seligy, V. L. (1988) Gene 70, 117–126
33. Risley, M. S., and Eckhardt, R. A. (1981) Dev. Biol. 84, 79–87
34. Brown, D. T., Alexander, B. T., and Sittman, D. B. (1996) Nucleic Acids Res. 24, 486–493
35. Gunjan, A., Alexander, B. T., Sittman, D. B., and Brown, D. T. (1999) J. Biol. Chem. 274, 37950–37956
36. Sun, J. M., Ali, Z., Lurz, R., and Ruiz-Carrillo, A. (1990) EMBO J. 9, 1651–1658
37. Sun, J.-M., Wiaderkiewicz, R., and Ruiz-Carrillo, A. (1989) Science 245, 68–71
38. Wright, J. M., Wiersma, P. A., and Dixon, G. H. (1987) Eur. J. Biochem. 168, 281–285
39. Misteli, T., Gunjan, A., Hock, R., Bustin, M., and Brown, D. T. (2000) Nature 408, 877–881
40. Dimitrov, S., and Wolffe, A. P. (1996) EMBO J. 15, 5897–5906
41. Hill, C. S., Rimmer, J. M., Green, B. N., Finch, J. T., and Thomas, J. O. (1991) EMBO J. 10, 1939–1948
42. Wagner, T. E., Hartford, J. B., Serra, M., Vandegeirt, V., and Sung, M. T. (1977) Biochemistry 16, 286–290
43. Shimizu, M., Shindo, H., Takahashi, K., Taniguchi, S., and Matsumoto, U. (1987) J. Biochem. (Tokyo) 102, 351–358
44. Cassidy, D. M., and Blackler, A. W. (1978) Dev. Biol. 63, 224–232
45. Sung, M. T., Hartford, J., Bundman, M., and Vidalakas, G. (1977) Biochemistry 16, 279–285
46. Cary, P. D., Hines, M. L., Bradbury, E. M., Smith, B. J., and Johns, E. W. (1981) Eur. J. Biochem. 120, 371–377
47. Grigoryev, S. A., Bednar, J., and Woodcock, C. L. (1999) J. Biol. Chem. 274, 5626–5636
48. Grigoryev, S. A., Solovieva, V. O., Spirin, K. S., and Krasheninnikov, I. A. (1992) Exp. Cell Res. 198, 268–275
49. Eufemi, M., Ferraro, A., Altieri, F., Cervoni, L., and Turano, C. (2000) Mol. Biol. Rep. 27, 183–189
50. Zlatanova, J., Caiafa, P., and van Holde, K. (2000) FASEB J. 14, 1697–1704
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