DNA repeat lengths of erythrocyte chromatin differing in content of histones H1 and H5¹

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ABSTRACT

Among the erythrocytes of chicken, trout, carp, and sucker, the relative proportion of the lysine-rich histone H5 varied from 20 to 0% of the total histones. Following digestion of nuclear chromatin with micrococcal nuclease, each of them displayed a longer DNA repeat length and greater repeat length heterogeneity than found in liver chromatin. Fish erythrocytes possessed similar repeat lengths of 207-209 base pairs which was 10-12 base pairs shorter than in chicken erythrocyte chromatin and approximately 10 base pairs longer than in liver chromatin. No correlation existed between the DNA repeat length or repeat length heterogeneity and the relative proportion of H5.

INTRODUCTION

Digestion by endogenous and exogenous nucleases first revealed the fundamentally repetitive nature of eukaryotic chromatin substructure (1,2,3). Discrete DNA lengths of approximately 140 base pairs were found to be associated with two each of the common histones H2A, H2B, H3, and H4, comprising the core of the regular repeating unit or nucleosome (4). These core particles are joined by shorter lengths of linker DNA with which the lysine-rich histones H1 and H5 may be associated (5,6).

Digestion of chromatin with micrococcal nuclease yields fragments that are multiples of a characteristic length of DNA including the core and adjoining linker. This repeat length varies among different species, different tissues of the same organism, different cell types of the same tissue, and even within a single cell type in different metabolic states (7-17). Since the nucleosome core has almost invariant DNA length among diverse chromatin sources, the variability in DNA repeat length most likely reflects differences of DNA length in the nucleosome linker (7,8,12).

It has been postulated that the length of linker DNA is correlated with differences in size or basicity of the lysine-rich histones, the most diver-

gent of histone classes (12,13). Chicken erythrocyte chromatin contains a major cell-specific histone H5 which may partially replace H1. It resembles H1 in many respects including richness in lysine, but differs in its higher content of arginine and serine (18). Since chicken erythrocytes show DNA repeat lengths elevated above those from tissues devoid of H5 (12), it was inferred that linker DNA associated with H5 is longer than that associated with H1.

To test this hypothesis, we measured DNA repeat lengths in erythrocytes and livers from chicken, trout, carp, and sucker. These erythrocytes vary in their relative proportions of H5 and H1 even to the extreme of an absence of H5 in sucker erythrocytes (19). Although DNA repeat lengths in brief digests of erythrocyte nuclei were longer than in liver, a direct correlation between the relative amounts of H5 and the length of the DNA repeat was not evident. Erythrocyte nuclear digests were notable for a greater heterogeneity of DNA repeats, with longer oligomers revealing shorter repeat lengths than smaller ones after prolonged digestion.

METHODS

Preparation of Nuclei

Rainbow trout (*Salmo gairdnerii*), carp (*Cyprinus carpio*), and white sucker (*Catostomus commersoni*) were bled from the caudal artery into ice cold Alsever's solution (20). Nuclei were prepared from erythrocytes lysed with 0.6% saponin in 0.32 M sucrose and 10 mM CaCl_2 , followed by washes in 0.32 M sucrose and 10 mM CaCl_2 until the supernatants were clear (21); 1.0 mM PMSF (phenylmethylsulfonyl fluoride) was included in all media before nuclease digestion (22). Chicken erythrocyte nuclei were obtained by methods described previously (23), except that nuclei were finally washed in a solution of 0.32 M sucrose, 10 mM CaCl_2 , and 1.0 mM PMSF.

Chicken and fish livers were washed and minced in solution A (0.32 M sucrose, 1.0 mM MgCl_2 , 2.0 mM CaCl_2 , 1.0 mM PMSF, and 10 mM Tris, pH 7.0), then homogenized for 45 seconds at medium speed in a Virtis "23" homogenizer. The homogenate was filtered through four layers of cheesecloth and adjusted to 0.05% (w/v) Triton X-100. Chicken liver filtrates were adjusted to 0.5% Triton X-100. These filtrates were homogenized by fifteen full passes by hand with a "loose-fitting" Dounce homogenizer and centrifuged at 2,500 rpm for 10 minutes at 4°C in a Beckman JA-20 rotor. The nuclei were washed in solution A and resuspended in 2.2 M sucrose with the same constituents as solution A. This suspension was layered over 10 mL of the same solution and

nuclei were pelleted at 26,000 rpm for 45 minutes at 4°C in a Beckman SW-27 rotor. Nuclei were again washed in solution A.

Nuclease Digestion

For nuclease digestion, nuclei were resuspended into 0.32 M sucrose, 0.5 mM CaCl₂, 1.0 mM Tris, pH 8.0 to a concentration of 10 OD₂₆₀/mL. After a preincubation of 37°C, digestion was initiated by adjusting the erythrocyte nuclear suspension to 2.4 units/mL micrococcal nuclease (Sigma Chemical) and the liver nuclear suspension to 6.0 units/mL. Digestion progressed at 37°C and aliquots were drawn at various digestion times. Digestion was inhibited by chilling on ice and by adjusting the suspension to 10 mM EDTA.

DNA was extracted from each sample by adjusting them to 1.0% SDS, 1.0 M NaCl, and by repeatedly extracting the aqueous phase with chloroform:isoamyl alcohol (24:1) (24). The aqueous phase was dialysed against cold 0.5 mM EDTA overnight and lyophilized.

Histone Extraction

Histones were extracted from nuclei twice with cold 0.25 N HCl and precipitated overnight in about ten volumes of acetone at -20°C. The precipitates were redissolved in distilled water and lyophilized.

Electrophoresis of DNA

The purified DNA fragments were compared by electrophoresis in slab gels (0.3 x 14 x 20 cm) composed of 2.5% acrylamide and 0.5% agarose. The running buffer was the Tris, acetate, EDTA system described by Loenig (25). Electrophoresis progressed for 4.5 hours at 80 volts and gels were stained with 20 µg/mL ethidium bromide for 15 minutes. Each was photographed under ultraviolet illumination onto Polaroid P/N film, utilizing a U.V. barrier filter. In some cases the negatives were scanned at OD₅₇₀.

Representative DNA fragments from each tissue were co-electrophoresed with T7⁺ Hind II restriction fragments (26) as size markers (Figure 1). The electrophoretic system calibrated DNA fragment lengths precisely according to size within the range required.

The oligomer DNA fragment lengths were plotted as a function of digestion time and their sizes at 0 minutes of digestion were determined by extrapolation. The DNA repeat length of initially digested chromatin was calculated as the slope of these fragment sizes as a function of oligomer number by linear regression analysis.

The band width for each of the DNA monomers and oligomers was measured at one-half the maximum peak height from scans of Polaroid negatives at OD₅₇₀.

Figure 1. Calibration curve for measuring DNA fragment lengths in base pairs. $T7^+$ Hind II restriction fragments (26) were electrophoresed through slab gels composed of 2.5% acrylamide and 0.5% agarose. The distance of migration was measured from photographic enlargements of ethidium bromide stained gels or from scans of the photographic negatives.

The breadth of each band was calibrated, in base pairs, from the curve in Figure 1. The band widths of $T7^+$ Hind II DNA restriction fragments similar in size to the oligomers or monomers were subtracted from the values of the band widths to correct for their tendency to diffuse as a function of migration distance.

Electrophoresis of Histones

Histones were compared on 15% acrylamide gels containing SDS (0.15 x 14 x 20 cm) with a 5% stacking gel (27). Lyophilized histones were dissolved in 2.5% SDS, 0.125 M Tris, pH 6.8, 5% 2-mercaptoethanol, 10% glycerol by boiling for 3.0 minutes. Gels were electrophoresed for 20 hours at 75 volts and stained overnight with 0.25% amido black in 30% methanol, and 7% acetic acid. After destaining with agitation in 30% methanol and 7% acetic acid, the gels were scanned at OD₅₇₀. Individual peak areas were determined from the scans by planimetry and converted to percentage areas below the peaks.

The apparent molecular weights of trout and carp H5 were estimated from their electrophoretic mobilities relative to chicken H5, carp H3, and trout

H2A whose molecular weights are known (28-31).

RESULTS

Lysine-Rich Histones of Erythrocytes and Liver

Histones H1 and H5 were efficiently separated from each other and from the other histones by gel electrophoresis in acrylamide slab gels containing SDS, as shown in Figure 2. The relative peak areas under densitometric scans of several samples, after simultaneous electrophoresis and staining in a single slab, could be compared without risk of technical vagaries. The peaks of H1 and H5 revealed variation among the erythrocytes of different species. While chicken erythrocytes contained much H5 and little H1, white sucker had a major H1 peak and no H5; carp and trout showed intermediate levels of the two types of lysine-rich histone relative to the other histones. The combined content of H1 and H5 in proportion to total histones was constant

Figure 2. Densitometric profiles of histones from chicken and fish erythrocyte and liver separated by SDS slab gel electrophoresis. Total histones were separated by electrophoresis on 15% acrylamide gels containing SDS (27) and stained with 0.25% amido black. Each channel was scanned at OD_{570} .

(Table 1), confirming a reciprocal relationship between these proteins in mature erythrocytes (19).

Slab gels containing SDS indicated other differences among fish histones (Figure 2). The apparent molecular weights of trout and carp H5, estimated as 20,300 and 19,700 daltons, respectively, were smaller than that of chicken H5, reportedly 21,500 daltons (28). This variability is consistent with differences in other properties of H5 species (19,33). The electrophoretic mobilities of H2A, H3, and H4 were the same on SDS gels regardless of the source, but fish H2B migrated faster than chicken H2B and overran H2A under these conditions. Similar differences in mobilities were observed in gels containing acid-urea (19), with both erythrocytes and livers.

Fish and chicken erythrocyte and liver histones are compared on SDS gels in Figure 2. Liver preparations contained little H5. There was some tissue-specific variation among H1 subfractions of carp and chicken. Whether these differences in lysine-rich histones among species and cell types are reflected by DNA repeat lengths in chromatin was examined by micrococcal nuclease digestion.

Micrococcal Nuclease Digestion of Erythrocyte and Liver Nuclei

In all species examined, the erythrocyte nuclei were more rapidly digested than the liver nuclei. Whether this was caused by intrinsic differences in the two nuclear types or by the different methods of nuclear isolation was not explored. For comparison, the concentration of micrococcal nuclease was adjusted to produce similar rates of digestion. The relative amounts of monomers and successive oligomers generated during digestion remained

Table 1. The relative peak areas of lysine-rich histones from erythrocytes after polyacrylamide-gel electrophoresis.

Species	Peak area* (% of total histone)		
	H5	H1	H5 + H1
White sucker	0	33	33
Carp	6	28	34
Trout	11	22	33
Chicken	21	10	31

*Uncorrected for specific colour yields of the respective histones.

comparable among all erythrocyte species.

As digestion of all nuclear types progressed, the proportion of short oligomers and monomers increased as expected and oligomer DNA lengths decreased as illustrated for carp erythrocyte nuclei in Figure 3. Measurements of these and analogous fragments from fish and chicken erythrocytes and livers were plotted as a function of time of digestion in Figure 4. All of the oligomer fragments were quickly digested to a limit length which remained constant despite the progressive shift from larger to smaller oligomers. However, when the DNA lengths of successive oligomers from erythrocytes are plotted as a function of oligomer number at different times of digestion (Figure 5), it is evident that the relationship is linear from

Figure 3. DNA fragments from progressively digested carp erythrocyte nuclei. Carp erythrocyte nuclei were digested for 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 minutes at 37°C, with 2.4 units/mL micrococcal nuclease. DNA fragments were purified and separated by electrophoresis as described in Figure 1. The indicated oligomer numbers are multiples of the approximate repeat length. T7⁺ DNA was digested with Hind II restriction endonuclease (26) and co-electrophoresed with the above DNA fragments.

Figure 4. Sizes of DNA fragments from progressively digested erythrocyte and liver nuclei. DNA fragments were purified from progressively digested erythrocyte and liver nuclei of fish and chicken at various digestion times and separated by electrophoresis as in Figure 1. The DNA fragment lengths were calibrated against T7⁺ Hind II restriction fragments, shown in Figure 1. The oligomer numbers corresponding to DNA fragments are indicated on each panel.

dimers to pentamers and hexamers only for digestions extrapolated to 0 minutes. From the slope of these lines at 0 minutes of digestion, the repeat lengths were determined for chicken, trout, carp, and sucker erythrocytes as well as chicken, trout, and carp livers (Table 2). Since insuffi-

Figure 5. Progressive digestion of carp erythrocyte nuclei. DNA fragment lengths in base pairs from Figure 4 are plotted against oligomer number at 1.0 min, 2.0 min, 8.0 min of nuclease digestion and 0 min obtained by extrapolation.

Table 2. Initial DNA repeat lengths in erythrocyte and liver chromatin.

Species	Erythrocyte	Liver
White sucker	207 $\pm$ 2	200*
Carp	209 $\pm$ 4	203 $\pm$ 2
Trout	207 $\pm$ 4	201 $\pm$ 5
Chicken	219 $\pm$ 3	197 $\pm$ 2

DNA repeat lengths, in base pairs, were determined at 0 minutes of digestion from linear regression analysis of DNA fragment lengths $\pm$ the standard error of the slope.

*Estimated by parallel comparison with carp liver.

ent points could be obtained for extrapolation using sucker liver nuclei, its repeat length was estimated by comparison with parallel samples from carp.

Three groups of DNA repeat lengths at 0 minutes of digestion are evident in the cells examined (Table 2). The livers from chicken and all three fish had similar repeat lengths, about 200 base pairs. DNA repeat lengths did not differ significantly among the erythrocytes of the three species of fish but all were nearly 10 base pairs longer than the corresponding livers. In contrast the repeat length in chicken erythrocytes was 219 base pairs, about 10 base pairs longer than in fish erythrocytes and 20 base pairs longer than in livers.

Heterogeneity of DNA Repeat Lengths within Erythrocytes

Relative heterogeneity of repeat lengths was indicated by two parameters. First, band width, in base pairs of DNA, increased from monomers to larger oligomers in erythrocyte nuclei. These band widths are corrected for diffusion by subtraction of the band widths of restriction fragments of homogeneous length and comparable size and are summarized in Table 3.

A disparity is also revealed between the DNA repeat lengths of small and large oligomers of nuclei after prolonged digestion (i.e., at least 16 minutes) (see Figure 5). This is summarized in Table 4 for four species of erythrocytes and three of liver.

Since these repeat lengths were obtained by subtraction of each oligomer fragment length from its larger neighbour, individual variations are emphasized. It is evident that repeat lengths in liver changed little after several minutes of digestion (Table 4 compared with Table 2). In contrast the repeat lengths of all erythrocyte DNA species decreased to levels which did not differ significantly among species but which showed heterogeneity

Table 3. DNA band widths of monomers and oligomers from fish erythrocytes.

Species	Oligomer number			
	1	2	3	4
White sucker	58	85	128	212
Carp	74	94	138	214
Trout	67	78	146	177

DNA band widths in base pairs were measured at half peak height of each DNA fragment after at least 4 minutes digestion with micrococcal nuclease.

Table 4. DNA repeat lengths in erythrocyte and liver chromatin.

Oligomer increment	Cell type	Species			
		White Sucker	Carp	Trout	Chicken
2-3/3-4	Erythrocyte	206 $\pm$ 3	213 $\pm$ 6	197 $\pm$ 4	208 $\pm$ 5
4-5/5-6	Erythrocyte	174 $\pm$ 2	166 $\pm$ 5	164 $\pm$ 8	182 $\pm$ 9
2-3/3-4/4-5	Liver	-	198 $\pm$ 4	206 $\pm$ 3	192 $\pm$ 30

DNA repeat length was measured in base pairs as the difference between successive fragment lengths $\pm$ standard error of the mean; number of measurements, $n = 4$ for erythrocyte, 5 or 6 for liver. The DNA fragments were generated by digestion of nuclei for at least 16 minutes with micrococcal nuclease.

related to size of oligomer. The repeat lengths between dimers, trimers, and tetramers averaged around 206 base pairs, while those between tetramers, pentamers, and hexamers were 30-40 base pairs shorter. From Figure 3 it is evident that the long repeat lengths in oligomers produced by short digestion of erythrocyte nuclei represent a minor fraction of the total DNA in chromatin, while the intermediate and short repeat lengths revealed by prolonged digestion reflect a major portion of the chromatin.

DISCUSSION

The nuclei of mature erythrocytes from nonmammalian vertebrates have mostly condensed chromatin in a genetically repressed state (35-38). A specific lysine-rich histone has been found in many but by no means all of these erythrocytes (18,19,23,39,40). In avian erythrocytes, the high level of H5 may be linked to the ultimate condensation of chromatin and repression of gene expression (41,42), but in chick embryonic erythrocytes the bulk of condensation is not correlated with H5 levels (43) and in regenerating goose blood a high rate of transcription (44) occurs despite normal levels of H5 (45).

In the bulk of the chromatin the variability in repeat lengths of partially digested DNA is supposed to arise from differences in lengths of nucleosomal linker DNA with which the lysine-rich histones H1 and H5 are associated (5-8,11,17). Furthermore it was suggested that the relatively long repeat length in digests from erythrocyte chromatin was correlated with the peculiar properties of the erythrocyte-specific lysine-rich histone (12), especially

as an increase in content of this histone is accompanied by an increase in DNA repeat length in chick embryonic erythrocyte chromatin (46). That this correlation is not general has been demonstrated by the relatively short repeat length in DNA from erythrocytes of *Sipunculus nudus*, which contains an H5-like histone (47) and the moderately short repeat length from regenerating erythroid cells of chicken (15), which show no appreciable difference in H5 content.

The observations presented here suggest that the elevated DNA repeat length in erythrocyte chromatin does not simply reflect the relative content of the cell-specific histone H5. The DNA repeat lengths of trout, carp, and sucker erythrocytes was essentially identical at 207-209 base pairs. This constancy among fish erythrocytes was not correlated with the relative content of H5 which ranged from zero to one-third of the lysine-rich histones. Chicken erythrocytes, in which H5 constitutes two-thirds of the lysine-rich histones, possess a DNA repeat length of 219 base pairs which is 10-12 base pairs longer than those of fish erythrocytes. The DNA repeat length for chicken erythrocytes reported here is somewhat longer than the 197-216 base pairs reported by others (7,8,12,15,48). The DNA repeat lengths of chicken and fish livers were 199-200 base pairs, which is shorter than those for erythrocytes, in agreement with published values for avian and mammalian livers (7,12,15,48). Our measurements were determined by extrapolation to 0 time of digestion, which selects for chromatin fractions with the longest repeat lengths. From these and published observations we conclude that DNA repeat length in erythrocyte chromatin may reflect the state of nuclear metabolic activity or cell maturity but not directly the content of particular lysine-rich histones.

The long DNA repeat length characteristic of chicken erythrocyte nuclei is put into perspective by the evident heterogeneity in digests of all species of erythrocytes. The 210-220 base-pair repeat quickly disappeared as this minor fraction of chromatin was preferentially digested to monomers. More prolonged digests of chicken and fish erythrocyte chromatin contained a mixture of oligomers in which the smaller, more homogeneous fragments clustered about 200-210 base-pair repeats, while the longer oligomers were comprised of heterogeneous populations averaging repeat lengths of 160-180 base pairs. Longer digestion might have eliminated some of the longer spacings leaving a residue of oligomers with short repeat lengths representing a substantial fraction of the total chromatin. While the occurrence of such heterogeneity may be characteristic of erythrocyte nuclei, it is not correlated with the content of H5, which varies greatly among these four species. Fur-

ther support for this conclusion was found in avian erythroid cells where repeat length heterogeneity remained unchanged despite a considerable accumulation of H5 (46). The extent of such heterogeneity appears to vary among the chromatins of diverse cell types and may be characteristic for each (8,17).

Heterogeneity of mononucleosomes has been attributed in part to the differential presence of H1 or H5 in erythrocyte chromatin fragments (49,50) containing DNA about 170, 180, and 200 base pairs in length. It is unlikely that such mononucleosomes influence repeat length heterogeneity in erythrocytes, particularly those of carp and sucker which possess little and no H5, respectively.

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