

Short communication

**ALLELIC ISOFORMS OF THE CHICKEN AND DUCK HISTONE H1.a**

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**Abstract:** Two isoforms of the erythrocyte histone H1.a were identified in two conservative flocks of Rhode Island Red chickens and six conservative flocks of ducks. The H1.a1 and H1.a2 isoforms formed three phenotypes (a1, a2 and a1a2) and were electrophoretically similar in the two species. The frequency of phenotype and histone H1.a allele occurrence varied within the genetic groups of birds, but the relatively rare allele  $a^2$  was only detected in chicken and duck strains with colored feathers. Using mass spectrometry, we established that the difference between the measured masses of the duck H1.a isoforms was 156 Da. Since this value corresponds to the mass of the arginine residue alone or to the combined mass of the valine and glycine residues, we believe that the polymorphism of duck histone H1.a might have originated from sequence variation. A mass difference of 1 Da observed between chicken H1.a isoforms corresponded well to the previously detected Glu/Lys substitution (0.9414 Da) at position 117.

**Keywords:** Chicken, Duck, Electrophoresis, Histone H1.a, Polymorphism, Mass spectrometry

**INTRODUCTION**

The vertebrate histone H1 family consists of a number of variants encoded by distinct genes [1]. The relative proportions of the histone H1 variants vary during development [2] and differentiation [3], and may depend on the tissue and species [4]. These properties suggest that some subtypes evolved to perform individual or specialized function [5, 6]. The H1 proteins of higher eukaryotes have a tripartite structure with a central globular domain, and basic N- and C-terminal tails that are unstructured in solution under physiological conditions [7].

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Abbreviations used: PMSF – phenylmethylsulfonyl fluoride; PVDF –polyvinylidene fluoride; SDS – sodium dodecyl sulfate

Previously, six H1 subtypes were detected in the polyacrylamide gel patterns of total histone H1 preparations from chicken and duck erythrocytes [8, 9]. The amino acid sequences of these proteins are highly conserved with differences between the subtypes localized preferentially to the N- and C-terminal domains [10]. Apart from the non-allelic variants, there are also allelic isoforms of certain H1 subtypes, as evidenced using electrophoretic [11, 12] and chromatographic [13, 14] procedures. Two allelic isoforms of the erythrocyte histone H1.a were found [8, 9] in chicken and duck populations. We recently used microsequencing techniques to identify a specific amino acid substitution in the chicken H1.a isoforms [9]. In this study, we performed a partial sequence analysis and molecular mass determination of duck histone H1.a isoforms to identify possible alterations to their C-terminal domain sequences.

## **MATERIALS AND METHODS**

### **Animals**

Rhode Island Red chickens (*Gallus gallus*) were bred as genetic reserve flocks on Szczytno farm near Dęblin (lines R55 and R11) and Chorzelów farm near Mielec (line R11) in Poland. The conservative flocks of ducks (*Anas platyrhynchos*) were obtained from the Dworzyska Waterfowl Breeding Station of the National Research Institute of Animal Production in Poland.

### **Isolation of erythrocytes, nuclei and histone H1**

Blood from individual chickens and ducks was collected separately into tubes containing SSC solution (0.15 M sodium chloride, 0.015 M sodium citrate) supplemented with 0.1 mM PMSF. The blood cells were pelleted by centrifugation. The supernatant with a layer of leukocytes was discarded, and the erythrocytes were washed twice with the SSC–PMSF solution. The washed erythrocytes were frozen at  $-20^{\circ}\text{C}$  until needed. Erythrocyte nuclei were isolated using saponin solution and histone H1 was extracted with perchloric acid as described previously [9].

### **Electrophoretic separations**

Protein preparations (1 mg) were dissolved in a sample buffer (200  $\mu\text{l}$ ) containing 8 M urea, 0.9 M acetic acid and 10% 2-mercaptoethanol (2-ME). Aliquots containing 25  $\mu\text{g}$  protein were then loaded into separate gel wells for electrophoresis. Histone H1 was separated on an acetic acid–urea polyacrylamide gel (15% acrylamide, 8 M urea and 0.9 M acetic acid) and on a two-dimensional polyacrylamide gel (first in acetic acid–urea gel and then in 15% polyacrylamide containing 0.1% SDS). Preparative SDS gel electrophoresis of individual histone H1 subtypes was performed as described elsewhere [9].

### **In-gel digestion, electroblotting and amino acid analysis**

Stained bands containing concentrated histone H1 subtypes were cut out from the SDS preparative gel and incubated in 0.1% SDS, 1 mM EDTA, 10% glycerol

and 0.125 M Tris/HCl (pH 6.8). Next, the samples were loaded into separate wells of the 15% polyacrylamide gel containing 0.1% SDS and overlaid with 4  $\mu$ l of  $\alpha$ -chymotrypsin (45 U/mg) dissolved in the above buffer at a concentration of 1  $\mu$ g/ $\mu$ l. After electrophoresis, the proteins were electroblotted onto PVDF membrane [9]. The membrane was stained with 0.1% Coomassie Blue R-250 and the chymotryptic C-peptide bands of histone H1.a were excised from the blots and analyzed via automated Edman sequencing. The N-terminal amino acid sequence analysis was performed on a Model 491 gas-phase sequencer (Perkin Elmer-Applied Biosystems) at the BioCenter facility of the Jagiellonian University, Kraków, Poland.

#### **Molecular mass determination via electrospray ionization mass spectrometry (ESI-MS)**

Protein and peptide bands were cut out from an SDS–polyacrylamide gel stained with zinc in an imidazole buffer containing SDS [15]. The gel slices were placed in separate polypropylene microcentrifuge tubes. Desalting in Tris-glycine buffer and further extraction of proteins and peptides with formic acid–water–2-propanol (1:3:2, v/v/v) was performed according to published procedures [16]. Prior to ESI-MS analysis, the extracts were desalted and concentrated using a 300  $\mu$ m i.d. (inner diameter) x 5 mm Micro-Precolumn Cartridge packed with 5  $\mu$ m C4 PepMap300 with 300 Å wide pores (LC Packings) and eluted with a 10-min linear gradient of acetonitrile (45–90%), containing 0.1% formic acid, at a flow rate of 0.0002 ml/min. Molecular mass determination of the proteins and peptides was performed on a Q-ToF I mass spectrometer (Waters Micromass) under an electrospray voltage of 2.5 kV. The ion spectra were deconvoluted using the MaxEnt.

## **RESULTS AND DISCUSSION**

### **Electrophoretic screening and population analysis**

Although the electrophoretic patterns of histone H1 from chicken and duck erythrocytes were similar, certain differences in the mobilities and intensities of the six protein bands or spots were observed when comparing the two species (Fig. 1). In duck, besides the common subtypes H1.a, .a', .b, .c, .c' and .d, an additional histone was found, H1.z, which has a slower electrophoretic mobility than that of H1.a (Fig. 1C and D) [11].

In the acetic acid–urea gel, two isoforms of erythrocyte histone H1.a (H1.a1 and H1.a2) were identified within two conservative flocks of Rhode Island Red chicken [9] and six conservative duck groups (Kh1, O1, KhO, SB, P3 and K2) [8]. On the basis of one- and two-dimensional gel patterns, we determined that the two H1.a isoforms had similar molecular masses and different net charges. In both species, they formed three phenotypes: a1, a2 and a1a2 (Fig. 1).

Homozygous birds with the a1 phenotype only had a single protein, H1.a1, while the heterozygous individuals with phenotype a1a2 had two separate isoforms, H1.a1 and H1.a2. The second homozygous phenotype, a2, was composed of

histone H1.a2 that co-migrated with a minor H1.a1-like protein. This protein spot, named H1.y in chicken, was also discernible in the gel pattern of total histone H1 from duck individuals with phenotype a2 (Fig. 1). The mode of inheritance of the histone H1.a phenotypes in duck [8] and chicken [9] suggested that a single gene with two co-dominant alleles at a locus is responsible for the observed polymorphism.

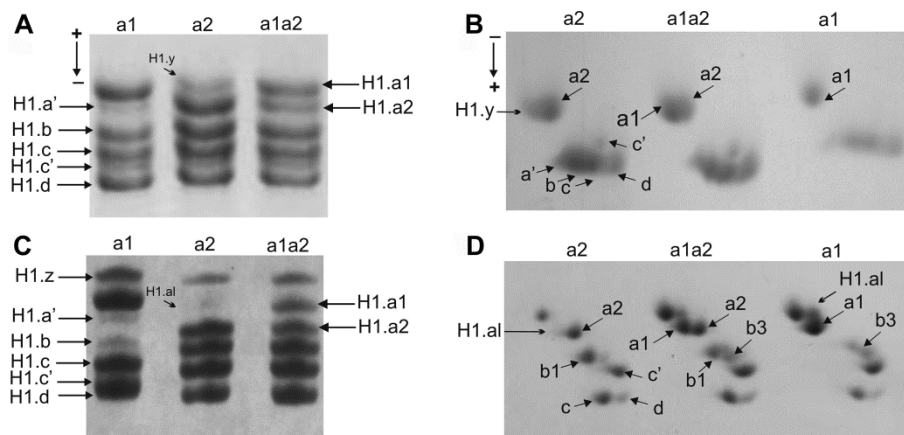


Fig. 1. A comparison of three phenotypes of erythrocyte histone H1.a in chicken (A and B) and duck (C and D). The erythrocyte histone H1 was resolved in acetic acid-urea (A and C) and two-dimensional polyacrylamide gels (B and D). The electrophoretic patterns of duck histone H1 (C and D) also show another polymorphic subtype, H1.b [28]. H1.z, .a, .a', .b, .c, .c' and .d are the subtypes of histone H1. H1.y is a chicken-specific protein band. H1.al is a duck H1.a1-like protein. Histone H1.a isoforms H1.a1 and H1.a2 were designated as .a1 and .a2, respectively. The phenotypes of histone H1.a are indicated as a1, a2 and a1a2 above the appropriate gel patterns.

Phenotype a1 occurred in all of the duck and chicken flocks tested [8, 9]. It was the only form of the H1.a in Peking-type ducks (P8 and P9) and broiler ducks (A1 and A2) and it predominated in both chicken lines and most duck strains, except in the brown-feathered Khaki Campbell ducks (Kh1 and KhO). Phenotype a2 was detected at low frequency (0.040–0.099) in three conservative groups of ducks with colored feathers (Kh1, O1, KhO) and in the P3 population. The conservative flocks R11 and R55 of Rhode Island Red chicken were enriched in phenotype a1 (0.825–0.983) with an admixture of rare phenotype a1a2 (0.017–0.175) [9]. The third chicken phenotype a2 was only found in the progeny of the heterozygous a1a2 individuals that were mated on purpose [9]. Thus, a rare allele  $a^2$  was found only in Rhode Island Red chickens with brownish-red plumage, in ducks Kh1 and O1, which have colored feathers, and in the populations derived from these groups (Fig. 2).

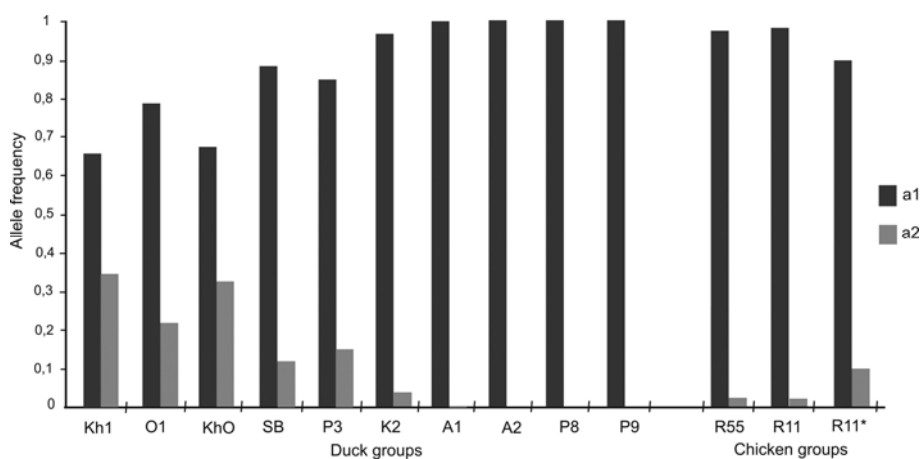


Fig. 2. The allele frequency of the erythrocyte histone H1.a in conservative groups of duck and chicken. Chickens from the same genetic group, R11, were bred at two farms: Szczytno near Dęblin (R11) and Chorzelów near Mielec (R11\*) in Poland.

#### Enzymatic digestion and mass spectrometry analyses

Using limited proteolysis with  $\alpha$ -chymotrypsin it has been shown [8, 9] that chicken and duck histone H1.a isoforms differed in their C-terminal peptides. A partial sequence comparison of chymotryptic C-peptides of chicken isoforms H1.a1 and H1.a2 revealed only one amino acid substitution, which is located at the beginning of the C-terminal region. A glutamic acid at position 117 in histone H1.a1 was replaced by lysine in histone H1.a2 [9]. Because the electrophoretic properties of the chicken and duck H1.a isoforms were very similar, we first sought to determine whether the Glu/Lys substitution could also occur in the duck H1.a. For this purpose, the C-terminal fragment produced by  $\alpha$ -chymotrypsin cleavage of duck histone H1.a was electroblotted onto PVDF membrane and analyzed using automated Edman degradation. The sequences for the first 20 amino acids in the chymotryptic C-peptides isolated from homozygous (a1 and a2) duck were compared with the same region of chicken histone H1.a affected by the E/K mutation (Fig. 3). The sequences of the analyzed H1.a fragments from ducks with phenotype a1 and a2 were identical and we did not find here the Glu/Lys substitution (Fig. 3).

Protein mass determinations are effective for studying both post-translational modifications and amino acid sequence variations [17], so we used electrospray ionization mass spectrometry to find differences in the molecular masses between duck H1.a isoforms. Fig. 4 shows deconvoluted profiles of histone H1.a obtained from duck phenotypes a1, a2 and a1a2. The main peaks in phenotypes a1 and a2 had molecular masses of 22332.5020 Da (isoform H1.a1) and 22176.252 Da (isoform H1.a2; Fig. 4A and B). As expected, two peaks with similar abundance and molecular masses of 22332.502 Da and 22176.502, respectively corresponding to the H1.a1 and H1.a2 isoforms, were observed in phenotype a1a2 (Fig. 4C).

110	117	129
Ch H1.a1	R L S K K P G <u>E</u> V K E K A P K K K A S A	
Ch H1.a2	R L S K K P G <u>K</u> V K E K A P K K K A S A	
Du H1.a1	R L S K K P G <u>E</u> V K E K A P K K <u>R</u> A A A	
Du H1.a2	R L S K K P G <u>E</u> V K E K A P K K <u>R</u> A A A	
Du H1.z1	R L S K K P G <u>E</u> V K E K A P K K <u>R</u> A A A	
Du H1.b1	R L S K K P G <u>E</u> V K E K A P K K <u>R</u> A A A	
1	8	17 20

Fig. 3. The alignment of the first 20 amino acid residues in the chymotryptic C-peptide obtained from the chicken histone H1.a1 (Ch H1.a1) and H1.a2 (Ch H1.a2) isoforms, the duck histone H1.a1 (Du H1.a1) and H1.a2 (Du H1.a2) isoforms, the duck histone H1.z1 isoform (Du H1.z1) and the duck histone H1.b1 isoform (Du H1.b1). Polymorphic chicken histone H1.a has two accession numbers deposited in UniProtKB, P08287 (for histone H1.a1 with E117) and P84553 (for histone H1.a2 with K117). The numbers above the alignment refer to the amino acid sequence of the chicken histone H1.a.

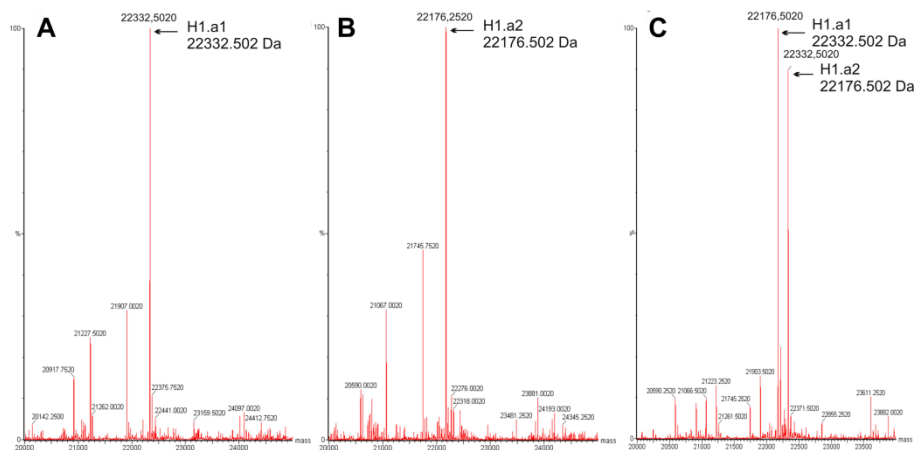


Fig. 4. Deconvolution plots of duck histone H1.a isoforms isolated from individuals of phenotype a1 (A), a2 (B) and a1a2 (C). The molecular masses determined by ESI MS method were 22332.502 Da (phenotype a1) and 22176.520 Da (phenotype a2).

Besides analysis of the full-length protein, we also estimated the molecular masses of chymotryptic N-terminal peptides derived from duck H1.a isoforms. The obtained plots (Fig. 5) indicated that the H1.a1 N-peptide (10704.251 Da) had the same molecular mass as the H1.a2 N-peptide (10704.251 Da). This data together with electrophoretic separation of  $\alpha$ -chymotryptic products of the H1.a isoforms [8] clearly proved that the C-terminal peptides should be responsible for the polymorphism of duck histone H1.a. However, we did not reveal any protein peak that corresponded to the predicted molecular masses of chymotryptic C-peptides of the duck H1.a1 and H1.a2 in the deconvoluted

spectra (Fig. 5). The lack of H1 C-peptides in the tested samples might result from their non-specific adsorption during sample manipulation. Table 1 summarizes the measured masses of H1.a isoforms and their chymotryptic N-peptides. The mass difference between the H1.a1 and H1.a2 proteins was estimated to be 156 Da. This value was too high to correspond to any single amino acid substitution. The biggest mass difference of 129.16 Da should result from Gly/Trp replacement ([www.expasy.org/tools/findmod/aa\\_subst\\_average.html](http://www.expasy.org/tools/findmod/aa_subst_average.html)).

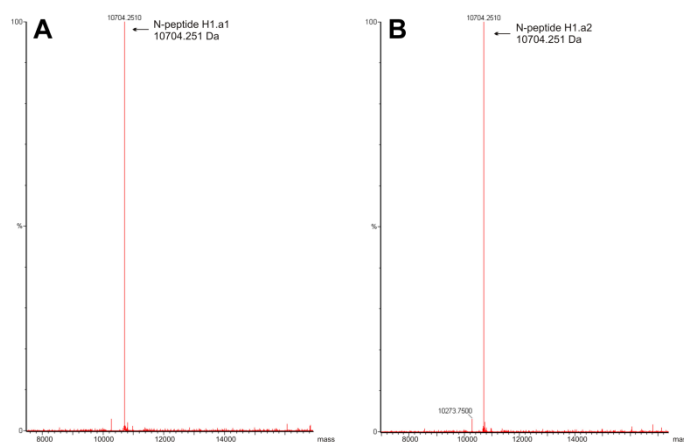


Fig. 5. Deconvoluted mass spectra of chymotryptic N-peptides derived from duck histone H1.a of phenotype a1 and a2. The molecular mass determined using the ESI MS method was 10704.251 for both histone H1.a phenotypes.

Table 1. Molecular masses of duck H1.a isoforms and their chymotryptic N-peptides estimated using the ESI-MS method

Phenotypes of histone H1.a	V H1.a isoforms	Measured mass (M)		$\Delta M$ of H1.a isoforms [Da]
		Intact protein [Da]	Chymotryptic N-peptide [Da]	
a1	H1.a1	22332.5020	10704.2510	
a2	H1.a2	22176.2520	10704.2510	156.25
a1a2	H1.a1	22332.5020		
	H1.a2	22176.5020		

Amino acid deletions and insertions and/or amino acid replacements [18] and post-translational modifications [19] may lead to structural variation at the protein level. The observed mass difference between duck H1.a isoforms (156 Da) corresponds to the molecular mass of arginine (156.1875 Da). This amino acid is present in the C-terminal domain (residues 127–218) in the duck histone H1 subtype of known sequence (UniProtKB, accession P09426). On the other hand, a deletion of valine (99.0648) and glycine (57.0215) residues at positions 116 and 118, respectively, in the duck histone H1 C-terminal domain might also

account for the observed mass difference between the allelic isoforms. However, a simultaneous deletion of two noncontiguous amino acid residues seems to be much less likely. Thus, the allelic isoforms of histone H1.a might have originated through a deletion or insertion of a single codon for arginine. As the identified mass difference between duck histone H1.a isoforms fits well to a dimethylated (14.01 Da for one methyl group) and/or formylated (27.994 Da) lysine residue, a post-translational modification may contribute to the variation within the histone H1.a as well. A minor protein spot H1.y accompanying histone H1.a in the two-dimensional gel patterns of total chicken and duck erythrocyte histone H1 (Fig. 1) could result from a modification affecting the charge of the protein.

In contrast to the H1.a duck polymorphism, we did not find significant mass differences between the main peaks in the samples of the chicken histones H1.a1 and H1.a2 (data not shown). These peaks have respective masses of 22438.5 Da and 22437.5 Da and corresponded to monoacetylated forms of H1.a1 and H1.a2. The difference between the measured masses of chicken H1.a1 and H1.a2 proteins was 1 Da and corresponded exactly to the mass difference between these allelic variants (0.9414 Da) with the Glu117Lys replacement [9].

A similar migration of duck and chicken histone H1.a isoforms in two-dimension polyacrylamide gels (Fig. 1B and D) suggested that these proteins possessed similar molecular masses. However, unlike the chicken histones H1.a1 and H1.a2, a significant mass difference between duck H1.a isoforms was revealed (Table 1). Although we did not find the same Glu/Lys substitution in duck histone H1.a, typical for H1.a polymorphism in chicken, our results suggested a presence of a putative change in the amino acid sequence in the C-terminal domain of the duck H1.a molecule. We believe that the duck histone H1.a2 allelic isoform could have originated through the deletion of a single arginine residue from the more prevalent isoform H1.a1.

Linker histones play an important role in chromatin fiber folding [20] and gene regulation [21, 22]. Several observations suggest that the carboxy-terminal domain (CTD) of histone H1 represents an intrinsically disordered region [23] that adopts a structured conformation upon binding to DNA [24]. The CTD of histone H1 is likely to be involved in competitive interaction with the chromatin and other nuclear proteins [25]. On the basis of these studies, it seems likely that the amino acid changes in the CTD region [26, 27] may affect its secondary structure and thereby the interaction of the mutated H1 variant with its targets.

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