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# Chromatin from two classes of platyhelminthes display both protist H1 and higher eukaryote core histones

Mario Galindo[a], Nelson Varela[a], Ingrid Espinoza[a], Gabriela Cecilia Toro[a], Ulf Hellman[b], Christer Wernstedt[b], Norbel Galanti[a,*]

[a] *Program of Cellular and Molecular Biology, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Casilla 70061, Correo 7, Santiago, Chile*
[b] *Ludwig Institute for Cancer Research, Box 595, SE – 751 24, Uppsala, Sweden*

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**Abstract Histones from the parasitic platyhelminthes, *Echinococcus granulosus* and *Fasciola hepatica*, were systematically characterized. Core histones H2A, H2B, H3 and H4, which were identified on the basis of amino acid sequencing and mass spectrometry data, showed conserved electrophoretic patterns. Histones H1, identified on the basis of physicochemical properties, amino acid composition and amino acid sequencing, showed divergence, both in their number and electrophoretic mobilities, between the two species and among other organisms. According to these data, core histones but not H1 histones, would be stabilized during evolution at the level of platyhelminthes.**


*Keywords:* Platyhelminth; Histone; Evolution; *Echinococcus*; *Fasciola*

## 1. Introduction

Histones were thought to be highly conserved in eukaryotes. However, these chromosomal proteins present a high degree of divergence in lower eukaryotes, such as *Entamoeba* [1], *Trichomonas* [2], *Trypanosoma* [3–8], *Leishmania* and *Crithidia* [9], dinoflagellates [10], *ciliata* [11] and *Giardia* [12]. Histone divergence during evolution is expressed at the amino acid sequence level in core histones and mostly at the structural level in H1 histones.

By contrast, intermediate eukaryotes, such as the nematoda *Caenorhabditis* [13,14] and *Ascaris* [15]; annelida *Platynereis* [16] and *Chaetopterus* [17]; and *Echiura* [18], have shown more conserved histones than those of lower eukaryotes.

Platyhelminthes are an ancient phylum with a basal phylogenetic position in Bilateralia [19]. We have characterized systematically the histones from representative species belonging to two different classes of the Platyhelminth phylum: *Echinococcus granulosus* (class Cestoda) and *Fasciola hepatica* (class Trematoda).

* Corresponding author. Fax: +56-2-737-3158.
*E-mail address:* ngalanti@med.uchile.cl (N. Galanti).

*Abbreviations:* TAU–PAGE, Triton DF16-acid–urea polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography

## 2. Materials and methods

### 2.1. Parasites

Protoscoleces of *E. granulosus* were collected from liver and lung cysts of naturally infected sheep. Adult worms of *F. hepatica* were collected from livers of naturally infected bovines.

### 2.2. Extraction of histones

Chromatin from *E. granulosus* and *F. hepatica* was obtained following Stein et al. [20] with modifications [3]. Histones were extracted from chromatin, as reported [3,4]. Protease inhibitors 5 mM phenylmethylsulfonyl fluoride, 2.5 mM tosyl-L-lysine chloromethyl ketone, 1 mg/ml leupeptin, 1 mg/ml antipain, 1 mg/ml trypsin inhibitor, and 1 mM ethylenediaminetetracetic acid were used throughout the whole procedure. Histones from *Trypanosoma cruzi*, sea urchin sperm cells and calf thymus, prepared as indicated above, were used as controls.

### 2.3. Purification of histone H1

Histone H1 was extracted and purified from *E. granulosus* and *F. hepatica* chromatin using the procedure of Sanders [21].

### 2.4. Polyacrylamide gel electrophoresis

Histones were analyzed by Triton DF16-acid–urea polyacrylamide gel electrophoresis (TAU–PAGE), as described [3,4].

### 2.5. HPLC fractionation and isolation of histones

Histone separation was also performed by reverse-phase high-pressure liquid chromatography (HPLC) on a 2.1 × 100 mm, 3 μm support (uRPC C2/C18 SC 2.1/10) from Amersham Biosciences, Uppsala, Sweden, as described [6]. The fractions eluted were analyzed by TAU–PAGE.

### 2.6. Amino acid composition analysis

E2 chromosomal protein from *E. granulosus*, purified by HPLC, was hydrolyzed as indicated [6]. The hydrolyzates were analyzed in a 4151 ALFa Plus I analyzer from LKB, Bromma, Sweden, following the instructions of the manufacturer. Amino acid compositions of histone H1 from *T. cruzi*, sea urchin and calf thymus were used as referents [6,22,23].

### 2.7. Protease digestion, separation of peptides, amino acid sequencing and sequence comparisons

Indicated bands of histones were cut out from TAU–PAGE gels and treated for in-gel digestion [24], with LysC protease from *Achromobacter lyticus*, WAKO, Neuss, Germany. After digestion, the peptides present in the reaction mixture were separated by reverse-phase HPLC, as previously described [24].

Selected peptides were sequenced by automated Edman degradation in an Applied Biosystems Protein Sequencer, model 494 A, operated according to the manufacturer's instructions. Each partial amino acid sequence was screened against the Protein Identification Resource of the National Biomedical Research Foundation with the FASTP program.

### 2.8. Mass spectrometry

Each peptide mixture obtained by protease digestion was subjected to peptide mass finger-printing by matrix assisted laser desorption

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ionization time of flight mass spectrometry. Aliquots of 0.3 μl from the digest were mixed with an equal volume of the matrix, saturated alfa-cyano 4-hydroxy cinnamic acid and applied to the steel target. Mass spectral data of the peptides were compared with a database of peptide mass values and the closest match to the unknown protein was identified. When peptides were used for amino acid sequencing, after tryptic in-gel digestion, the digest was adsorbed onto a ZipTip C18 and Lys residues were converted to homoarginine. Then, peptides were N-terminally sulfonated with the CAF-kit (Amersham Biosciences, Uppsala, Sweden) for improved Post Source Decay fragmentation using MALDI TOF/TOF (Ultraflex, Bruker Daltonics, Bremen, Germany) as described [25]. The exclusive y-ion fragment series generated allowed for manual sequence interpretation.

## 3. Results

### 3.1. *Electrophoretic characterization of* E. granulosus *and* F. hepatica *histones*

Fig. 1 shows the electrophoretic pattern of histones obtained from *T. cruzi* (lane 1), *E. granulosus* (lane 2), *F. hepatica* (lane 3), sea urchin (lane 4) and calf thymus (lane 5) in a TAU–PAGE gel.

In *E. granulosus* and *F. hepatica*, six chromosomal proteins electromigrated in the range of those of protists or higher eukaryotes (Fig. 1, lanes 2 and 3). They were named as E1 through E6 and F1 through F6, from top to bottom. Proteins E1, E3, E4 and E5 from *E. granulosus* and F1/F2, F3/F4, F5 and F6 from *F. hepatica* showed electrophoretic mobilities similar to those observed for histones H2A, H3, H2B and H4 from sea urchin and calf thymus (Fig. 1, lanes 2–5). Besides, protein E2 from *E. granulosus* has an electrophoretic mobility similar to that of histones H1 from sea urchin and calf thymus (Fig. 1, lanes 2, 4 and 5), while protein E6 showed a high electrophoretic mobility, similar to that of histone H1 of *T. cruzi* (Fig. 1, lanes 1 and 2). In *F. hepatica*, proteins with electrophoretic mobilities similar to those of histones H1 from sea urchin, calf thymus or *T. cruzi* were not observed (Fig. 1, lane 3).

### 3.2. *Characterization of histones H1 from* E. granulosus *and* F. hepatica *by their solubility properties*

Histones H1 from different organisms are preferentially extracted from chromatin in 0.75 M perchloric acid and purified from this extract by differential precipitation in acid-acetone [6,9,12,25]. By using this procedure, we obtained proteins E2

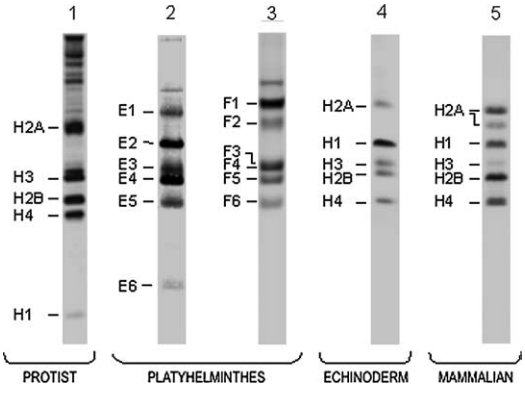


Fig. 1. PAGE of histones of *T. cruzi* (lane 1), *E. granulosus* (lane 2), *F. hepatica* (lane 3), sea urchin (lane 4) and calf thymus (lane 5). Technical conditions were as described [3,4. Histones from *T. cruzi*, sea urchin and calf thymus were used as standard. Histones of *E. granulosus* and *F. hepatica* were named from top to bottom in the gel as E1, E2, E3, E4, E5, E6 and F1, F2, F3, F4, F5 and F6, respectively.

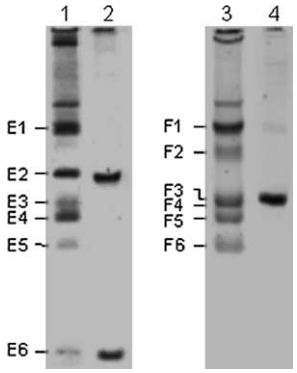


Fig. 2. H1 histones of *E. granulosus* and *F. hepatica*. Histones H1 of *E. granulosus* (lane 2) and of *F. hepatica* (lane 4) were extracted as described [3,4] and analyzed in a TAU–PAGE gel. Lane 1: *E. granulosus* histones; lane 3: *F. hepatica* histones.

and E6 from *E. granulosus* (Fig. 2, lanes 1 and 2) and F3 from *F. hepatica* (Fig. 2, lanes 3 and 4).

### 3.3. *Isolation of histones H1 of* E. granulosus *by reverse-phase HPLC*

Histones H1 from different organisms are eluted before the core histones when subjected to reverse-phase HPLC fractionation [6,9,12,26]. Fig. 3 shows the separation of histone fractions from *E. granulosus*, *T. cruzi* and calf thymus, using this procedure. In *E. granulosus*, two peaks with low retention times were observed (Fig. 3A, peaks I and II). When these subfractions were separately analyzed in TAU–PAGE gels, only proteins E6 and E2 were observed in peaks I and II, respectively (Fig. 3A, lanes I and II). These proteins were eluted at the range of the gradient defined by the phases of elution of histones H1 from both *T. cruzi* (Fig. 3B, peak I, lane I) and calf thymus (Fig. 3C, peaks I and II, lanes I and II). Consequently, proteins E2 and E6 from *E. granulosus* and protein F3 from *F. hepatica* (not shown) present HPLC elution profiles similar to those of histone H1 from other sources and are the same as those that were purified by the Sanders procedure (Fig. 2, lanes 2 and 4).

### 3.4. *Amino acid composition analysis of E2 chromosomal protein from* E. granulosus

Table 1 shows the amino acid composition of protein E2 from *E. granulosus*, in comparison to the amino acid composition of histone H1 from *T. cruzi*, sea urchin and calf thymus.

The basic/acid and the lysine/arginine ratios, as well as the percentage of hydrophobic amino acids of E2, correspond to those for histones H1 from the three species used as comparison.

### 3.5. *Identification of core histones and histones H1 by proteomic analysis*

After Edman degradation of selected peptides, proteins E1, E3, E4 and E5 from *E. granulosus* were identified by sequence matching as histones H2A, H3, H2B and H4, respectively (Table 2). Similarly, proteins E2 and E6 (middle sequence) were identified as histones H1 (Table 2). Applying mass spectrometry to the generated peptides, proteins F2, F4, F5 and F6 of *F. hepatica* were identified as histones H2A, H3, H2B and H4, respectively (Table 3). Using peptides for amino acid sequencing by y-ion fragment series generation and manual sequence interpretation, proteins E6 (Table 2, upper and lower sequences) and F3 (Table 3) were identified as hi-

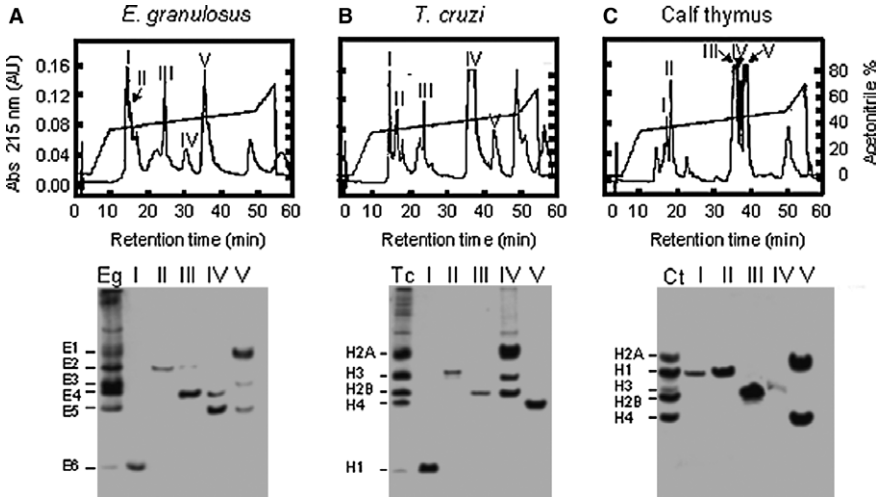


Fig. 3. Separation of histones from *E. granulosus* by reverse-phase HPLC. Histones from *E. granulosus* (A), *T. cruzi* (B) and calf thymus (C) were separated as indicated under Section 2. Histones and fractions (roman numbers) obtained after the HPLC fractionation were subjected to TAU–PAGE. Eg, Tc and Ct stand for histones from *E. granulosus*, *T. cruzi* and calf thymus, respectively.

Table 1
Comparative amino acid composition of E2 protein from *E. granulosus* with histone H1 from other organisms

| Amino acid[a] | *T. cruzi* H1 [6][b] | *E. granulosus* E2 | Sea urchin H1 [22][b] | Calf thymus H1[23][b] |
|---|---|---|---|---|
| Lys | 32.3 | 20.6 | 28.6 | 27.3 |
| His | 1.3 | 1.3 | 0.8 | – |
| Arg | 2.0 | 4.3 | 10.5 | 2.1 |
| Asp | 3.7 | 3.8 | 1.6 | 2.6 |
| Thr | 1.7 | 2.5 | 2.0 | 6.0 |
| Ser | 5.9 | 8.8 | 7.7 | 6.9 |
| Glu | 2.6 | 4.9 | 2.0 | 4.7 |
| Pro | 10.9 | 7.1 | 8.1 | 8.4 |
| Gly | 4.3 | 7.5 | 4.0 | 6.7 |
| Ala | 26.5 | 21.1 | 25.4 | 23.4 |
| Cys | – | – | – | – |
| Val | 4.7 | 10.5 | 3.6 | 6.0 |
| Met | – | – | 1.6 | – |
| Ile | 0.8 | 2.9 | 0.8 | 0.8 |
| Leu | 1.5 | 1.9 | 2.0 | 3.9 |
| Tyr | 0.7 | 1.5 | 0.8 | 0.6 |
| Phe | 0.5 | 1.3 | 0.4 | 0.6 |
| Basic/acid | 5.6 | 3.0 | 10.9 | 4.0 |
| Lys/Arg | 16.5 | 4.8 | 2.7 | 13.0 |
| % Hydrophobic | 49.2 | 52.3 | 45.2 | 49.8 |

[a] Amino acid composition of histone H1 from different organisms (references in brackets).
[b] Purificated by HPLC as in Fig. 3A.

stones H1. Protein F1 of *F. hepatica* was identified as vitelline (data not shown).

## 4. Discussion

*Trypanosoma cruzi* core histones presented divergent amino acid sequences and dissimilar electrophoretic mobilities when compared to higher eukaryotes [3–5,8]. These characteristics of *T. cruzi* core histones are quite likely related to the failure of its chromatin to condense into chromosomes [27]. On the contrary, core histones of *E. granulosus* and *F. hepatica* seem to be conserved with respect to their electrophoretic mobilities, which may be associated to the fact that these parasites condense their chromatin during cell division [28,29].

Core histones of intermediate eukaryotes, such as *Caenorhabditis elegans*, *Platynereis dumerilii* and *Urechis caupo*, also showed histones H3 and H4 similar to those of their vertebrate homologs [13,14,16,18]. Interestingly, the gene sequence of histone H4 from the parasitic platyhelminth *Schistosoma mansoni* is identical to the histone H4 gene of *Drosophila melanogaster* [30].

Taking together all this information, we postulate that core histones became conserved during evolution from the Platyhelminthes onwards, thus pointing to an early stabilization of these chromosomal proteins in metazoan.

Protein F1 was identified as vitelline, which is a structural component of the eggshell in *F. hepatica*. It is a basic protein [31], which explains its co-extraction with histones. Similarly, *Giardia lamblia* proteins giardins beta and gamma, which are rich in lysines, were co-extracted with the histones of this parasite [12].

Histone H1 of calf thymus is of about 21 kDa, containing three domains: amino-terminal, globular, and carboxy-terminal. Histone H1 of *T. cruzi*, in contrast, corresponds to a family of proteins of about 7.4–9.7 kDa [7], presenting only the carboxy-terminal domain. It is conserved regarding the amino acid sequence of its carboxy terminal, but it is structurally not conserved. Furthermore, protists show a highly variable structure in histone H1, from a very short protein in Trypanosome [7] to a more conserved structure in *Giardia* [12], Chlorophytes and Mycetozoa [32].

The amino acid composition of the chromosomal protein E2 from *E. granulosus* showed a high percentage of lysine and the proportions of alanine and proline expected for a histone H1. The percentage of lysine (20.6%) is in the same range as the one observed in *Schistosoma japonicum* histone H1 (24.6%) (Accession No. AAP06509). Consequently, the amino acid composition analysis of E2 clearly shows that this chromosomal protein corresponds to a histone H1. Furthermore, this protein was unequivocally identified as a histone H1 by amino acid sequencing. These data, and the electrophoretic mobility of E2 corresponding to that of histones H1 from higher eukaryotes, strongly suggest that E2 is a conserved histone H1.

Table 2
Identified histones in *E. granulosus*

| Band[a] | Peptide sequence[b] / Histone sequence[c] | | Histone identified[d] | Accession number[e] |
|---|---|---|---|---|
| E1 | KTRIIPRLAQL<br>76 KTRIIPRHLQL 86 | (*Hs*) | Histone H2A | NP_060737 |
| E2 | VAATAASVSK<br>13 VAATPASVEK 22 | (*D*) | Histone H1 | BAC54554 |
| E2 | PVAVI SAQA<br>5 VVAV–SAS P 12 | (*D*) | Histone H1 | P17268 |
| E3 | KSTELLIRK<br>57 KSTELLIRK 65 | (*Hs*) | Histone H3 | NP_002098 |
| E3 | KLPFQRLVRRIAQNF<br>65 KLPFQRLVREIAQDF 79 | (*Hs*) | Histone H3 | NP_002098 |
| E4 | KHMSIMNSFV<br>58 KAMGIMNSFV 67 | (*Hs*) | Histone H2B | NP_003509 |
| E5 | KTVTAMDVVYALK<br>80 KTVTAMDVVYALK 92 | (*Hs*) | Histone H4 | NP_003529 |
| E6 | AGLQF-PRR<br>1-MLRFVPRR 8 | (*Cf*) | Histone H1 | 2206467C |
| E6 | RXTK<br>23 RSTK 26 | (*C*) | Histone H1 | AAB52426 |
| E6 | FEPLLXLNVK<br>69 FRKLLLLN LK 79 | (*A*) | Histone H1 | CAA44312 |

[a] Identifier of the corresponding bands as in Fig. 1.
[b] Amino acid sequences of peptides generated by LysC.
[c] Amino acid sequences of histones from *Homo sapiens* (*Hs*), *Drosophila* (*D*), *Crithidia fasciculata* (*Cf*), *Caenorhabditis* (*C*) and *Arabidopsis* (*A*).
[d] Histone showing highest score of alignment.
[e] Accession numbers of the corresponding sequences in [c].

Table 3
Identified histones in *F. hepatica*

| Band[a] | MALDI mass (Da)[c] | Histone residues[d] | Matched peptide[e] | Delta mass | Histone identified[h] | Accession number[i] |
|---|---|---|---|---|---|---|
| F2 | 943.52 | 22–30 | AGLQFPVGR | 0.00 | Histone H2A | CAA16944 |
| | 836.41 | 37–43 | KGNYAER | 0.00 | (17% of the protein) | |
| | 849.51 | 83–89 | HLQLAIR | – | | |
| F4 | 1031.58 | 42–50 | YRPGTVALR | −0.01 | Histone H3 | HSHU3 |
| | 1027.62 | 66–73 | LPFQRLVR | −0.01 | (25% of the protein) | |
| | 1334.68 | 74–84 | EIAQDFKTDLR | | | |
| | 714.40 | 124–129 | DIQLAR | | | |
| F5 | 1167.58 | 48–58 | QVHPDTGISSK | −0.01 | Histone H2B | Q99880 |
| | 732.37 | 74–90 | IASEASR | −0.01 | (32% of the protein) | |
| | 900.49 | 81–87 | LAHYNKR | | | |
| | 815.45 | 94–100 | EIQTAVR | | | |
| | 952.59 | 101–109 | LLLPGELAK | – | | |
| F6 | 1324.74 | 25–36 | DNIQGITKPAIR | −0.01 | Histone H4 | HSHU4 |
| | 1335.71 | 46–56 | RISGLIYEETR | 0.00 | (31% of the protein) | |
| | 988.57 | 61–68 | VFLENVIR | | | |
| | Peptide sequence[f] / Histone sequence[g] | | | | | |
| F3 | VPATHPPVLDMLR<br>32 RAATHPPVIDMIG 44 | | | | Histone H1 | S09388 |
| F3[b] | LGPHLR<br>76 LGPHVR 81 | | | | Histone H1 | S09388 |

[a] Identifier of the corresponding bands as in Fig. 1.
[b] Purified by the Sander's procedure as in Fig. 2.
[c] Mass value of peptides generated by trypsin.
[d] Histone residues matching to peptide mass values.
[e] Amino acid sequence of *Hs* histone.
[f] Amino acid sequences of peptides generated by trypsin.
[g] Amino acid sequence of *Parechinus* histone H1.
[h] Histone showing highest alignment of match.
[i] Accession numbers of the corresponding sequences in [e] and [g].

On the other side, E6 displayed solubility properties and elution profiles in HPLC proper of a histone H1. Its high electrophoretic mobility in acid–urea PAGE is similar to the one observed for *T. cruzi* H1 histone. Furthermore, its amino acid sequencing is consistent with a histone H1. We then propose that *E. granulosus* presents conserved core histones, but a H1 histone (E2) similar to that of higher eukaryotes and other histone H1 (E6) showing similarities to those of protists.

Thus, in *E. granulosus*, a histone H1 (E2) displaying an electrophoretic mobility similar to that of vertebrate H1 coexists with another histone H1 (E6) similar to the one found in different Kinetoplastids and ciliates (Fig. 1, lanes 1, 2 and 5) [3,9,33]. Consequently, E2 would be organized by three domains as histone H1 from mammals, and E6 would be constituted mainly by the carboxy-terminal domain, as histone H1 from *T. cruzi* [7] and other protists [32]. Interestingly, a H1 histone displaying only the carboxy-terminal domain was described recently in the nematode *C. elegans* [34].

Protein F3 of *F. hepatica* was identified as histone H1 by amino acid sequencing. It shows an electrophoretic mobility close to the core histone H3 of vertebrates (Fig. 1, lanes 3 and 5). Similar results were found in the ciliates Tetrahymena [35] and Euplotes [36], and in the kinetoplastid Crithidia [9]. Specifically, histone H1 from ciliates displays a bipartite structure with one domain homologous to the H1 carboxy-terminal domain and another domain showing low similarity to the H1 globular-terminal domain (Histone data base: http:/genome.nhgri.nih.gov/histones/ and [32]).

Taking into account the evolutionary conservation of the carboxyl terminus in H1 histones, from protists to mammals [32,37] and the observation that the globular domain is relatively well conserved through evolution in animals, plants, and fungi [32], the electrophoretic behavior of histone H1 from *F. hepatica* (F3) may be the result of missing amino acid residues mainly in its amino terminal domain. Indeed, H1 histones displaying a conserved globular domain, a short carboxyl-terminal domain and an almost absent amino-terminal domain, were described in the annelid *P. dumerilli* [38]. These H1 histones show electrophoretic mobilities between histones H2B and H4 [39].

Differences in the electrophoretic mobilities of H1 histones from *E. granulosus* and *F. hepatica*, two classes of the platyhelminth phylum, are as evident as those observed between H1 histones of three genera of the Trypanosomatidae family [9], suggesting that in platyhelminthes, as well as in protists, H1 histones present several structural alternatives.

Regarding histone H1 from *F. hepatica*, it presents characteristics similar to some ciliates or to polychaeta histones H1. This postulate points to the platyhelminthes as presenting, at the level of chromatin structure, transitional molecular characteristics not only between protozoa and the rest of the eubilaterials, but also between themselves.

Finally, our results support the view that the structure of core histones was fixed early in the evolution while the structure of histone H1 was not, thus reinforcing the theory of a different evolutionary history for core histones and histone H1 during the evolution of eukaryotes [12,32,37].

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