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Chromatin and Histones From *Giardia lamblia*: A New Puzzle in Primitive Eukaryotes

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Abstract The three deepest eukaryote lineages in small subunit ribosomal RNA phylogenies are the amitochondriate Microsporidia, Metamonada, and Parabasalia. They are followed by either the Euglenozoa (e.g., *Euglena* and *Trypanosoma*) or the Percolozoa as the first mitochondria-containing eukaryotes. Considering the great divergence of histone proteins in protozoa we have extended our studies of histones from Trypanosomes (*Trypanosoma cruzi, Crithidia fasciculata* and *Leishmania mexicana*) to the Metamonada *Giardia lamblia*, since *Giardia* is thought to be one of the most primitive eukaryotes. In the present work, the structure of *G. lamblia* chromatin and the histone content of the soluble chromatin were investigated and compared with that of higher eukaryotes, represented by calf thymus. The chromatin is present as nucleosome filaments which resemble the calf thymus array in that they show a more regular arrangement than those described for *Trypanosoma*. SDS-polyacrylamide gel electrophoresis and protein characterization revealed that the four core histones described in *Giardia* are in the same range of divergence with the histones from other lower eukaryotes. In addition, *G. lamblia* presented an H1 histone with electrophoretic mobility resembling the H1 of higher eukaryotes, in spite of the fact that H1 has a different molecular mass in calf thymus. *Giardia* also presents a basic protein which was identified as an HU-like DNA-binding protein usually present in eubacteria, indicating a chimaeric composition for the DNA-binding protein set in this species. Finally, the phylogenetic analysis of selected core histone protein sequences place *Giardia* divergence before *Trypanosoma*, despite the fact that *Trypanosoma* branch shows an acceleration in the evolutionary rate pointing to an unusual evolutionary behavior in this lineage. J. Cell. Biochem. 82: 573–582, 2001.

Key words: Protozoa; *G. lamblia*; histones; chromatin; accelerated evolutionary rate; H1 histone

Giardia lamblia a flagellate protozoan pathogen of human and other mammals, is a major cause of enteric disease worldwide. This parasite infects the upper intestine of some 200 million people annually [Upcroft and Upcroft, 1998]. In addition to its medical importance, *Giardia* is considered as an excellent system for the study of evolution of fundamental cellular processes since it probably represents the earliest branch of the eukaryote lineage [Cavalier-Smith, 1993]. Sequence analysis of 16S-rRNA [Sogin et al., 1989, Wheelis et al., 1992, Van Keulen et al., 1993], elongation factor E1α [Hashimoto et al., 1994] and other molecular markers, show that *G. lamblia* is, in evolutionary terms, closer to the prokaryotes than other eukaryotes. *G. lamblia* is anaerobic and one of the best-characterized examples of one of the most primitive eukaryotes [Thompson et al., 1993].

Based on prokaryotic-like characteristics, Sogin et al. [1989] and Cavalier-Smith [1993] proposed that the Metamonada (including the diplomanad *Giardia)* are primitive. However

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due to certain features, they appear to represent a surviving relict of an early stage in eukaryote evolution.

Gillin et al. [1996] and Luján et al. [1995, 1997, 1998] have shown that in spite of being an early-diverging eukaryote, that has been considered to have no typical Golgi apparatus, it is a complex and highly developed cell. It is now clear that *G. lamblia* contains in its nucleus genes for all three of the mitochondrial heat-shock proteins, Hsp 10, Hsp 60 [Roger et al., 1998] and Hsp 70 [Gupta et al., 1994]. These Hsps are the most reliable tracers of eubacterial ancestry of the mitochondrion, and all three *Giardia* Hsps match firmly with mitochondrial Hsps in phylogenetic analyses. *G. lamblia* is amitochondrial, however, it seems that *Giardia* lost its mitochondria in the course of becoming an obligate parasite and that subsequent evolution has been reductive rather than acquisitive [Hashimoto et al., 1998, Roger et al., 1998]. These considerations have led investigators to propose that the common ancestor of all extant eukaryotes contained mitochondria and was thus aerobic. *Giardia*, as for higher eukaryotes, has a cytoskeleton [Crossley and Holberton, 1983], nuclear envelope [Friend, 1996], linear chromosomes capped with telomeric repeats and telomere positional regulation of gene expression [Le Blancq et al., 1991]. However, it is clear that it has primitive anaerobic bacterial features not present in more evolved eukaryotes.

Analysis of nuclei from *G. lamblia* trophozoites shows that each cell presents two apparently identical, transcriptionally active nuclei that divide at about the same time [Kabnick and Peattie, 1990]. Using cytological techniques 4–8 major *Giardia* chromosomes are evidenced, which are maintained for generation after generation in each of the nuclei [Kabnick and Peattie, 1991; Erlandsen and Rasch, 1994]. On the other hand, pulsed-field separation of intact chromosomes demonstrated the presence of at least five distinct sets of chromosomes in addition to the occurrence of more faintly staining minor bands of DNA [Adam, 1991]. Densitometric scanning suggested that each trophozoite contains ~30–50 chromosomal DNA molecules [Adam et al., 1988]. This data, and that from other investigators have been related with the *Giardia* ploidy with conflicting results. However, it appears that chromosome rearrangements [Le Blancq, 1994] and genome plasticity involving a number of chromosomes is usual in *G. lamblia* and do vary from strain to strain [Upcroft et al., 1993; Upcroft et al., 1996]. Prior to our study, we were not aware that any data about chromatin existed for this parasite, but core histone genes has been reported recently [Wu et al., 2000].

It is a well established fact that in all higher eukaryotes so far investigated, the basic structural unit of chromatin is the nucleosome, an octamer of the histones H2A, H2B, H3, and H4. Around this protein core are wrapped 166 base pairs of DNA [Luger et al., 1997; Santisteban et al., 1997]. The fifth histone, H1, is thought to be associated with 10 bp at each end of the DNA superhelix. At low ionic strength, the nucleosomes form, together with the linker DNA, the so-called 10 nm fiber [Crane-Robinson, 1997].

In this report we have shown the ultrastructural organization of *G. lamblia* chromatin, which was examined in a spread preparation [Miller and Bakken, 1972]. Micrographs illustrate that the basic chromatin fiber in *G. lamblia* exists in typical nucleosomal conformations. Coincidently, the DNA pattern typical of higher eukaryotes was obtained after digestion with micrococcal nuclease. Core histones and H1 histone were extracted and systematically characterized by electrophoresis and identified by amino acid sequencing or by mass spectrometry. An HU protein, like the HU-DNA-binding protein from Eubacteria was also found, indicating a new trait showing the chimaeric composition of this species. Finally, we have discussed the evolutionary behavior of histones from diverse origin. *Giardia* histones behave as other protist histones and *Trypanosoma* histones show an unusual feature, the acceleration of amino acid substitution rate suggesting a relaxation of natural selection in this lineage.

METHODS

Cell Culture

Trophozoites of the *G. lamblia* isolate WB clone 1267 were cultured in TYI-S-33 medium supplemented with 10% newborn calf serum and 0.5 mg/ml bovine bile as described by Keister [1983]. Cells were routinely harvested by centrifugation at Day 3 of culture when a confluent monolayer was observed, washed three times in phosphate buffered saline (PBS), and stored at −86°C in the presence of protease inhibitors phenyl methyl sulfonyl fluoride (PMSF) and tosyl-L-Lysine chloromethyl ketone (TLCK).

Chromatin Spreading

Chromatin spreads were essentially prepared according to the procedure of Miller and Bakken [1972]. Cells from monolayer growing cultures were harvested by centrifugation and washed in PBS. Cell pellets were lysed in PBS/1% NP-40, and the chromatin dispersed by incubating for 5, 15, and 30 min at room temperature. Aliquots of 100 μl were centrifuged through a 1 M sucrose cushion containing 10% formalin, pH 9.0, onto colloidon-carbon coated grids (400 μm mesh). The samples were dried with Photoflo, stained using an ethanolic solution of 4% phosphotungstec acid and viewed in a JEOL E-109 electron microscope.

Production of Soluble Chromatin Fragments

The chromatin was extracted and treated with micrococcal nuclease according to Savic et al. [1981] for 10 min. Then, the sample was treated with RNAse and proteinase K and the DNA fragments extracted. The digested product was analyzed in agarose gels 1.5%, SDS 0.1% as described by Weintraub [1984]. The same procedure was used for preparation of the calf thymus control.

Extraction of Histones

Chromatin was obtained following the procedure of Stein et al. [1975] with some modifications [Toro and Galanti, 1988]. Histones were extracted from chromatin as reported by Panyim and Chalkley [1969]. Five mM PMSF and 2.5 mM TLCK were used through out the whole procedure. The same procedure was applied to obtain histones from calf thymus and *T. cruzi* histones.

Purification of Histone H1

H1 histone was extracted from *G. lamblia*, calf thymus, and *T. cruzi* chromatin using the procedure of Sanders [1977], which was designed for the purification of H1 histones from different sources. Results were analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Polyacrylamide Gel Electrophoresis

Total histones and H1 histone purified by the Sanders procedure were analized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laenmli [1979], with the modifications of Thomas and Kornberg [1975].

Protease Digestion and Separation of Peptides

Indicated bands of *G. lamblia* proteins obtained in the migration region of control histones were cut out from SDS-PAGE gels and treated for in gel digestion [Hellman, 2000]. After digestion, the peptides present in the reaction mixture were separated by reversed-phase chromatography within 80 min at a flow rate of 100 μl/min at room temperature, with a linear acetonitrile gradient. The eluate was monitored at 215 nm [Toro et al., 1993].

Amino Acid Sequencing

Selected peptides obtained by digestion with protease Lys C and separated by HPLC were sequenced by automated Edman degradation in an Applied Biosystem Proteins Sequencer, Model 494 A. The instrument was operated according the manufacturer's instructions.

Sequence Comparisons

Each partial amino acid sequence obtained was screened against the proteins identification resource (PIR) of the National Biomedical Research Foundation with the FASTP program.

Mass Spectrometry

Peptide mixtures obtained by digestion with Lys C protease were subjected to peptide mass figer-printing by MALDI-TOF-MS (matrix assisted laser desorption ionization time of flight mass spectrometry). Aliquots of 0.5 μl from the digest were used for mass spectrometry analysis. 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.

Phylogenetic Analysis

Protein sequence alignments were performed using CLUSTAL W [Thompson et al., 1994]. Only sites without gaps were used for calculating the distance matrix (not shown). Phylogenetic trees were constructed by the neighbour-joining method [Saitou and Nei, 1987].

RESULTS

Chromatin Structure

The structure of soluble chromatin of *G. lamblia* is similar to that of higher eukaryotes. Figure 1 shows the chromatin spreads for 5 min, 15 min, and 30 min, fixed and prepared for

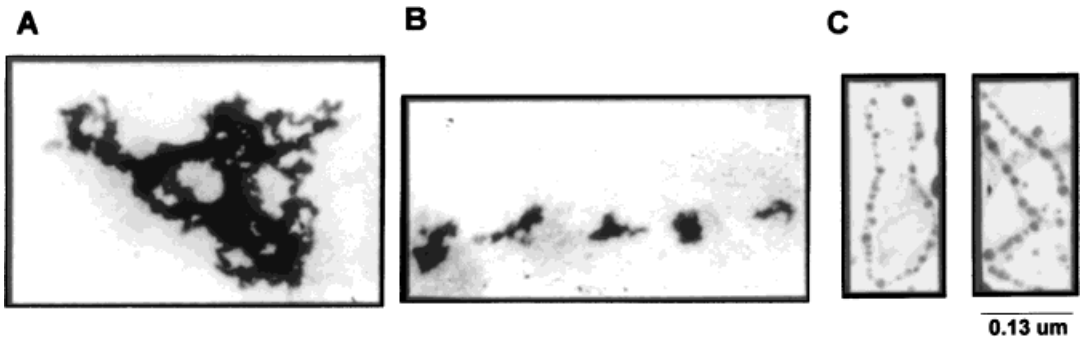

Fig. 1. Chromatin of *Giardia lamblia* observed under TEM, after treatment in dispersion solution. (**A**) 5 min, (**B**) 15, min and (**C**) 30 min. Note the discrete transition between higher-order chromatin fibers and the regular beaded fiber.

electron microscopy. Material consisting of densely packed fibers (Fig. 1A and B) are presented. At 30 min, the chromatin assumed a continuous "beads-on-a-string" aspect typical of that in higher eukaryotes (Fig. 1C). In this nucleosome-like particle a diameter of 10 nm was observed.

Analysis of Chromatin Digests

To evaluate the nucleosomal structure, *G. lamblia* chromatin was obtained and digested with micrococcal nuclease under the same conditions used to digest the thymus chromatin. DNA fragments generated by the nuclease were analyzed by gel electrophoresis. They formed a regular pattern of bands, in which the fragments were multiples of two, three, four, etc. times the smallest unit size, similar to the pattern obtained from calf thymus used as control and a form common to almost all the higher eukaryotes (Fig. 2, lanes 2 and 3). The unit size of ~200 bp is observed for both samples. This result confirms the regular beaded arrangement of *G. lamblia* chromatin obtained with TEM (Fig. 1C).

Figure 2 also shows that calf thymus chromatin without treatment with micrococcal nuclease (lane 1) is degraded by the endogen nuclease, which is activated at the calcium concentration in the digestion solution used [Savic et al., 1981]. In *G. lamblia* this does not occur (lane 4) meaning that the endogen nuclease is either not present at all, or it is inactive under the experiment conditions used.

Electrophoretic Characterization

Figure 3 shows the SDS electrophoretic pattern of histones obtained from calf thymus (lane 1) and *G. lamblia* (lane 2) in an SDS gel. These proteins were cut and digested with Lys C, and peptide mixture was analyzed by mass spectrometry, or separated by HPLC, and some of them were sequenced by automated Edman degradation.

These proteins were clearly identified by sequence comparisons as histones H1, H2B, H2A; or by mass comparisons as histone H4 as shown in Table I. The analysis performed with H4, allows us to conclude that two peptides

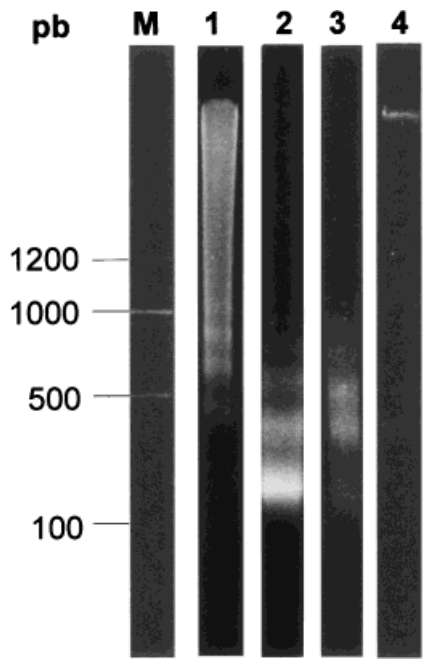

Fig. 2. Agarose gel electrophoresis of the resulting DNA fragments when calf thymus chromatin (**lane 2**) or *G. lamblia* chromatin (**lane 3**) is subjected to digestion with micrococcal nuclease for 10 min. The fragments form a pattern of discrete bands with a unitary size of ~200 bp. Lanes 1 and 4 correspond to chromatin from calf thymus and *G. lamblia,* respectively, incubated in digestion buffer for 10 min without micrococcal nuclease.

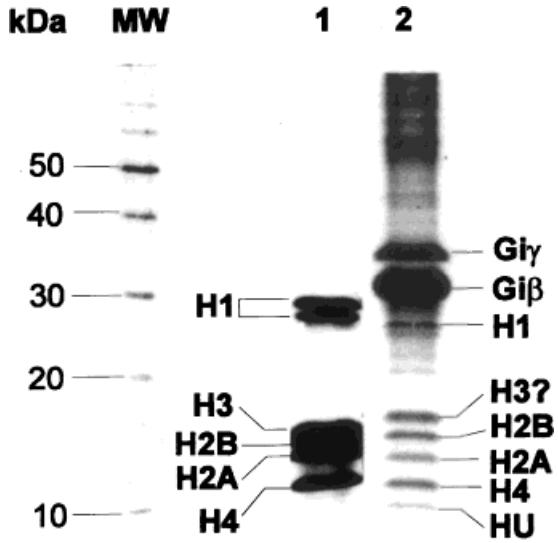

Fig. 3. SDS-PAGE electrophoresis of histones from calf thymus and *G. lamblia*. **Lane 1**: Calf thymus histones; **lane 2**: *G. lamblia* histones. Gi γ and Gi β: giardin γ and β respectively; HU: DNA-binding protein; M: molecular weight markers.

(32–44 and 80–91) are identical to those from human H4. The band named H3? in lane 2 from Figure 3, was not identifiable by these methods, it possibly corresponds to histone H3 considering its electrophoretic mobility as compared to H3 from calf thymus.

Three other proteins were identified; giardins γ and β (by mass spectrometry) and the histone-like protein HU by sequence comparisons, one of the most abundant DNA-binding proteins associated with the bacterial chromosome.

To investigate the possible H1 histone from *G. lamblia* we used the Sanders procedure, where H1 histone is preferentially extracted from chromatin. Figure 4 (lanes 1, 2, and 3) shows total histones, H1 and remnant histones after H1 extraction, respectively, from calf thymus, used as control. Lanes 4, 5 show total histones and H1 histone from *T. cruzi*. Lanes 6, 7, and 8 show total histones, H1 histone, and remnant histones without H1 from *G.lamblia*, respectively. Minor proteins present in total chromatin (lanes 1 and 6) are overrepresented in the remnant proteins (lanes 3 and 8) after H1 histone extraction.

Alternatively, when total histones from *G. lamblia* are fractionated by HPLC, the histone identified as H1 by the Sanders technique is eluted in the first peak, (not shown) [Triana, 1999]. This characteristic is common for H1 histones of other species [Toro et al., 1993]. Sequence analysis of one peptide obtained from digestion of this protein with Lys C, shows an identity of 67% with H1 histone from chicken (Table I).

Finally, phylogenetic trees from H2A, H2B, H3, and H4 histone protein sequences compiled are presented in Figure 5. The trees were rooted in unresolved trichotomies involving *Giardia*, flagellates, and all other lineages (histone H2A), *Giardia*, *Entamoeba*, and all other lineages (histone H2B), *Giardia*, *Entamoeba* + flagellates, and all other lineages (histone H3), and *Entamoeba*, flagellates and all other lineages (histone H4), respectively.

DISCUSSION

The parasitic protozoan *G. lamblia* belongs to the earliest identified lineage among eukaryotes and may therefore offer a unique insight

TABLE I. Protein Identification by Amino Acid Sequence or by Mass Spectrometry

Amino acid sequences	Mass spectrometry		
	Giardin γ (19% of the protein)		
	Giardin β (30% of the protein)		
H1			
(12) VAAPPTPAKAAP(23) chicken			
VAATPVSTKAAP *Giardia lamblia*			
H2B			
(100) LLLPGEL (106) Human			
LVIPGEI *Giardia lamblia*			
H2A			
(77) RIIPRHLQLAIRN (89) human			
RIVPNHILTALRK *G. lamblia*			
	Histone H4 (25% of the protein)		
	Mass (Da)	from residue	until residue
	1448.71	32	44
	1309.50	80	91
HU			
IEIPASKVPAFK *Bacillus subtilis*			
TIEIPESNVPAFK *Giardia lamblia*			

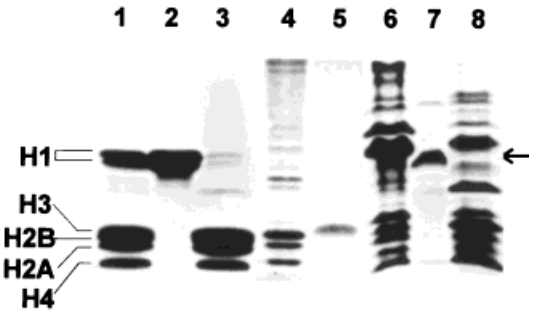

Fig. 4. Purification of *G. lamblia* H1 histone. All samples were analyzed by SDS-PAGE as described under Methods. **Lane 1**: Calf thymus histones; **lane 2**: Calf thymus histone H1 purified by the method of Sanders; **lane 3**: calf thymus remaining histones after extraction of H1 histone; **lane 4**: Histones from *T. cruzi*; **lane 5**: H1 histone from *T. cruzi*; **lane 6**: *G. lamblia* histones; **lane 7**: *G. lamblia* histone H1 purified by the Sanders technique; **lane 8**: *G. lamblia* histone proteins remaining after the extraction of H1 histone. The arrow shows the position of H1 in *Giardia lamblia* (lane 7) and its absence (lane 8).

into the progression from primitive to more complex eukaryote cells [Luján et al., 1995]. To our knowledge, this study provides the first description of chromatin nucleosomal structure, presence of histone H1, and presence of an HU in *Giardia*.

Protist chromatin organization show some characteristic features [Rizzo, 1985; Gorovsky, 1986; Wu et al., 1986; Toro and Galanti, 1988, 1990; Fodinger et al., 1993; Aslund et al., 1994; Bontempi et al., 1994; Galanti and Toro, 1994; Binder et al., 1995; Hecker et al., 1995; Soto et al., 1997; Galanti et al., 1998] which are different from that of higher eukaryote cells. For example, *Trypanosoma cruzi* and *Trypanosoma brucei* have a chromatin, which is physically and enzymatically fragile [Rubio et al., 1980; Hecker and Gander, 1985] compared to that of vertebrates. To obtain the classical pattern of 200 bp DNA fragments observed in nucleosomes from higher eukaryotes, a low concentration of nucleases and a short period of incubation is required [Rubio et al., 1980]. However, chromatin from *G. lamblia* presents the classical pattern of 200 bp DNA, under the same conditions of enzyme concentration and incubation time used for calf thymus, (Fig. 2) rather than being fragile as the chromatin of Trypanosomes.

Core histones are among the most conserved proteins in higher eukaryotes, however they present divergence in lower eukaryotes [Gorovsky, 1986]. In Trypanosomes an acceleration in the evolutionary rate of amino acid sustitution of histones is observed in relation to other eukaryotes [Toro et al., 1992], pointing that the high degree of conservation assumed in the amino acid sequence of histones seems to be valid only from fungi to mammals [Toro et al., 1992; Thatcher and Gorovsky, 1994]. In this case, *G. lamblia* presents core histones in the same range of divergence as other protists [Wu et al., 2000].

Protist histone divergence seems to be related to chromatin structure during the cell cycle. In vitro Trypanosome chromatin, at 10–100 mM salt concentrations, present poor condensation of nucleosome filaments and no compact fibers are formed, while rat chromatin presented solenoids [Hecker et al., 1995]. On the other hand, during mitosis Trypanosome chromatin does not condense into chromosomes [Solari, 1980]. This may, in part, be explained because the great divergence found in protist histones is located in the tails of each histone, specially in H3 and H4 and, according to Luger et al. [1997] histone amino-terminal tails contact neighbouring particles. On the other hand, *G. lamblia* condense chromatin [Kabnick and Peattie, 1991; Erlandsen and Rasch, 1994] in 4–8 dense aggregates of chromatin, which are maintained for generation after generation. In both *Giardia* and *Trypanosoma*, the nuclear envelope persists during cell division and the microtubular spindle apparatus is situated inside the nuclear membrane [Solari, 1980; Gillin et al., 1996].

With the methodology used, it was not possible to identify the H3 histone and that it was probably the protein showing migration close to H2B. Considering this migration, H3 histone from *G. lamblia* must have a molecular mass greater than H3 from calf thymus. This result agrees with the work of Wu et al. [2000] which shows that the H3 coding region from *G. lamblia* is longer than the H3 coding region from other species. Moreover, all peptides found by us in *G. lamblia* histones H_2A, H_2B and H_4 are also present in the amino acid sequences inferred from these genes as described by Wu et al. [2000].

In the present work, we showed for the first time the presence of the H1 histone in *Giardia lamblia*. This protein migrates near to calf thymus H1. If we consider that H1 from Trypanosomes are much smaller than H1 histones from higher eukaryotes, with only the C-terminal domain of their homologues expressed

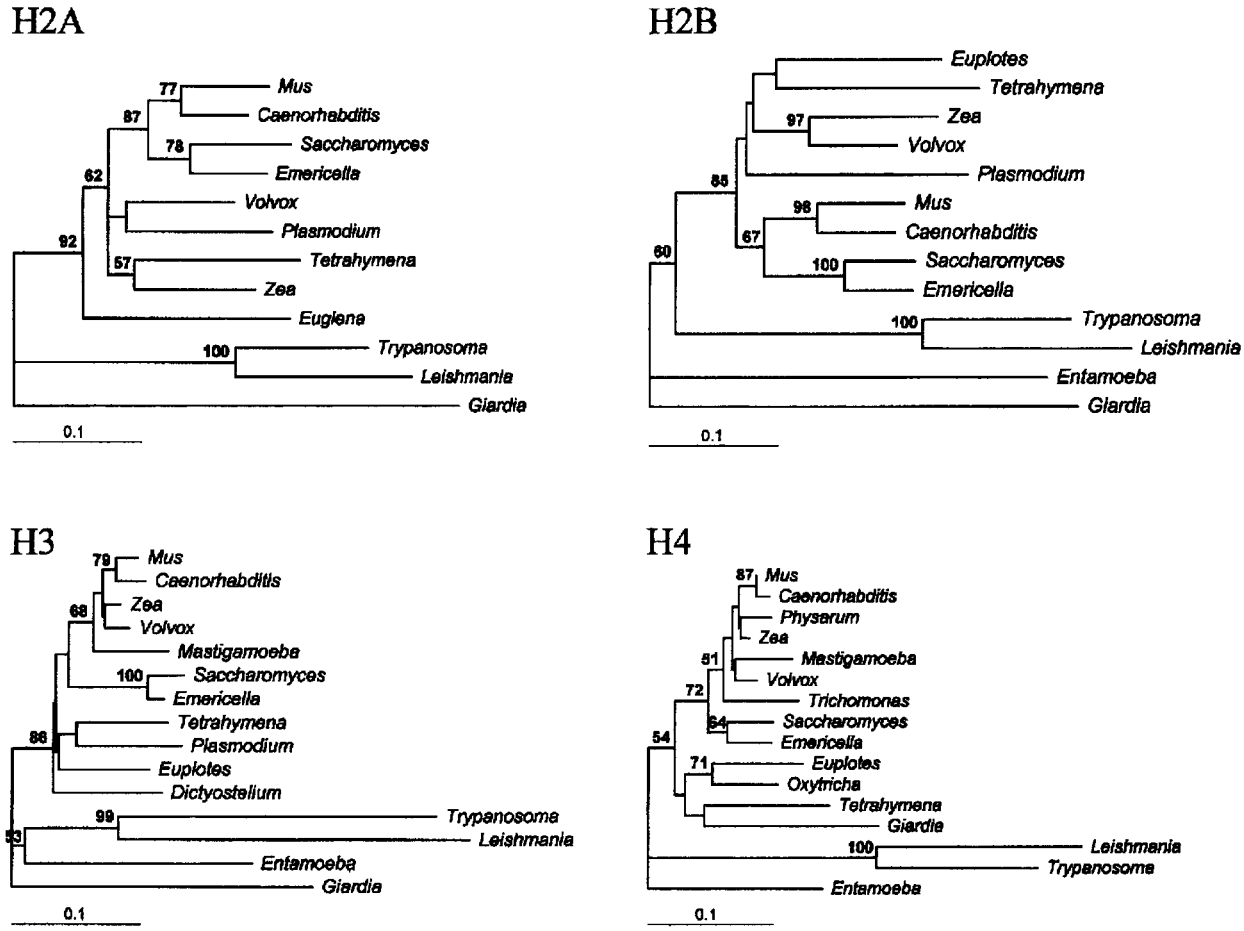

Fig. 5. Neighbor-joining tree of H2A, H2B, H3, and H4 histone proteins based upon 113, 101, 124, and 96 sites respectively, aligned without gaps. The bootstrap values given at the nodes are from 1,000 replicates. Only values above 50% are indicated. GenBank accession numbers are as follows; H2A histone; *Mus musculus,* S04152; *Caenorhabditis elegans,* AAF98222; *Saccharomyces cerevisiae,* NP_010511; *Emericella nidulans,* A27332; *Volvox carteri,* JQ0794; *Plasmodium falciparum,* P40282; *Tetrahymena thermophila,* S41471; *Zea mays,* T02076; *Euglena gracilis,* X73147; *Trypanosoma cruzi,* X67287; *Leishmania donovani,* CAA42652; *Giardia intestinalis,* AF139873; H2B histone *Euplotes crassus,* AAC47754; *Tetrahymena thermophila,* P08993; *Zea mays,* P54348; *Volvox carteri,* JQ0797; *Plasmodium falciparum,* AAC46612; *Mus musculus,* P10854; *Caenorhabditis elegans,* AAF98230; *Saccharomyces cerevisiae,* NP_010510; *Emericella nidulans,* P23754; *Trypanosoma cruzi,* P27795; *Leishmania major,* AAF04632; *Entamoeba histolytica,* AB002724; *Giardia intestinalis,* AF139874; H3 histone: *Mus musculus,* S06743; *Caenorhabditis elegans,* AAF98226; *Zea mays,* S57626; *Volvox carteri,* S00940; *Mastigamoeba balamuthi,* AAF00588; *Saccharomyces cerevisiae,* NP_014367; *Emericella nidulans,* S11938; *Tetrahymena thermophila,* S41499; *Plasmodium falciparum,* AAC46613; *Euplotes crassus,* AAB39721; *Dictyostelium discoideum,* U76664; *Trypanosoma cruzi,* AAA61706; *Leishmania major,* CAB55516; *Entamoeba histolytica,* L02418; *Giardia intestinalis,* AF139875; H4 histone: *Mus musculus,* S03426; *Caenorhabditis elegans,* AAF98223; *Physarum polycephalum,* X00449; *Zea mays,* HSZM4; *Mastigamoeba balamuthi;* AAF00589; *Volvox carteri,* S00939; *Trichomonas vaginalis,* X98017; *Saccharomyces cerevisiae,* NP_014368; *Emericella nidulans,* S11939; *Euplotes crassus,* AAB39722; *Oxytricha nova,* JS0154; *Tetrahymena thermophila,* A25875; *Giardia intestinalis,* AF139876; *Leishmania major,* CAB98434; *Trypanosoma cruzi,* AI035084; *Entamoeba histolytica,* S52262.

[Toro et al., 1993; Aslund et al., 1994; Espinoza et al., 1996; Toro, 2001], it is interesting that H1 histone from a protozoan, which had been considered more primitive than Trypanosomes presents a molecular mass in the range of the higher eukaryotes. This might be the consequence of a mutation which might induce a evolutionary alteration of *T. cruzi* H1 histone transcription [Toro, 2001]. Thus, in protists of primitive origin, different forms of H1 histones are present.

However, since H1 histone plays a key role in stabilization of the nucleosome and in formation of the chromatin higher order structure, it is not

surprising that a regularly array of the 10 nm chromatin fiber is found in *G. lamblia*, more similar to calf thymus chromatin than to the less arranged fiber of Trypanosomes [Hecker et al., 1995].

Considering the plasticity of the genomes, lateral gene transfer might be able to explain the presence of HU, typical of prokaryote organization [Claret and Rouviere-Yaniv, 1997] in the *Giardia lamblia* chromatin. Alternatively, HU may be present as a remnant of some eubacterial symbiont which had disappeared, and which might also explain the presence of the genes for Hsp 10, Hsp 60, and Hsp 70 in the genome of *Giardia*.

We also found giardins γ and β. These proteins from the cytoskeleton of *Giardia* are very rich in lysines [Crossley and Holberton, 1983], which might explain their solubility in H_2SO_4 and co-extraction with histones.

In this work we presented, for the first time, the nucleosomal structure of *G. lamblia* chromatin, the presence of a H1 histone and an HU-DNA binding protein. Additionally, the identification of their core histones in an SDS gel by amino acid sequencing of the protein or by mass spectrometry is also presented.

G. lamblia shows a chimaeric composition, with histones showing similarity to eukaryotes and with a DNA-binding protein homologous to the HU from eubacteria, leaving us once again with an intriguing puzzle to unravel.

Finally, according to the phylogenetic trees shown in Figure 5, *Giardia*, *Entamoeba*, and flagellate histones diverged earlier than histones from all other eukaryotes included in our comparison. These are clustered in a well-supported clade in all trees, with the exception of H4 histone tree, where *Giardia* is clustered with ciliates. However, this last clustering is not well supported by bootstrap values. In general, as a result of its low evolutionary rate and the small number of sites included, bootstrap values are low for most H4 histone tree nodes. In H4 histone and, specially, H3 trees, *Trypanosoma* and *Leishmania* have very long branches, despite the fact that, according to multiple evidences, they are close relatives. The large distance between these two species exclude the possibility of rooting the tree within their branch and points, instead, to an important difference in evolutionary rate between flagellate H3 and H4 histones and all other eukaryote histones, as noted before [Toro et al., 1992]. Animal (*Mus*, *Caenorhabditis*) histones seem to have, on the other hand, the slowest evolutionary rate. These opposing conditions have a clear correlation with differences in DNA compaction. *Entamoeba* and *Giardia* histones are, according to their evolutionary rate, in an intermediate condition and, as we mentioned before, they seem to have diverged earlier. The most parsimonious interpretation of all these differences is that *Entamoeba* and *Giardia* histone evolutionary rate is the primitive condition and that there has been an increase in structural restrictions (an increase in negative selection) in animal histone lineage and a relaxation of natural selection in the flagellate lineage. So, *Trypanosoma* and *Leishmania* H3 and H4 histones might be unusual, not because they are primitive, but because they are derived.

Why is natural selection relaxed in Trypanosome histone lineage? Is the HU protein a lateral gene acquisition from bacteria or is it a remnant from an endosymbiotic event in which *Giardia* acquired a mitocondria and later lost it? Is *G. lamblia* a chimaera produced by the endosymbiosis of an eubacteria in a nucleated eukaryote? We need to know more about these organisms to know fully about the pivotal role they seem to play in the evolutionary history of eukaryote cells.

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