

Isolation, characterisation and organisation of histone H1 genes in African trypanosomes

Eric Grüter · Bruno Betschart

Abstract A tandemly arranged gene family coding for histone H1 in African trypanosomes is described. Many variants, differing by some substitutions and/or deletions in their monotone and repetitive amino acid sequences, are found. The different variants can be sorted into three subtypes using their N-terminal region. PCR amplification experiments with primers specific to these three H1 subtypes suggest that the genes may be separated into two transcriptional units. Heterogeneity among species, subspecies and even strains was found. The transcripts are polyadenylated and the trans-splicing site is located very closely to the start codon. The intergenic regions are typical when compared to other polycistronic clusters described in trypanosomes. Amino acid sequence motifs may explain differences seen in chromatin compaction patterns between African and American trypanosomes.

Introduction

In trypanosomes, the nuclear genome exhibits some peculiar aspects that tend to demonstrate a particular form of DNA processing. No real chromatin condensation takes place and no chromosomes can be visualised at any stage of the cell-cycle or the parasitic life-cycle (Vickerman and Preston 1970). Despite this, core histones are all present and they assemble in a way similar to that found in higher eukaryotes to form the nucleosome core

particle. However, these histones do present some differences at the level of their primary structure, biochemical properties and relative nucleosomic positioning when compared to their counterparts in higher eukaryotes (Hecker et al. 1995; Galanti et al. 1998). Histone H1 has long been thought to be absent in trypanosomes, explaining the absence of real chromatin condensation. It was later described successively in several lower eukaryotes (Johmann and Gorovski 1976; Duschak and Cazzulo 1990; Fasel et al. 1994) including American (Toro et al. 1993) and African trypanosomes (Schlimme et al. 1993). Their small size, particular biochemical properties and weaker interaction with chromatin explain their late discovery, being either lost or hidden by the other histones when using standard isolation techniques.

In the African trypanosome *Trypanosoma brucei*, the causative agent of sleeping sickness and Nagana, the biochemical properties and structural functions of histones as well as chromatin assembly have been extensively studied (Hecker et al. 1994). These data showed that H1 is necessary for chromatin condensation and the regular spacing of nucleosomes. However, even by using techniques adapted to the weaker stability of H1 and core histones, the typical 30 nm chromatin fibre found in higher eukaryotes is never formed. Compaction is most pronounced in *Trypanosoma cruzi*, followed by *T. brucei brucei* bloodstream forms, while procyclic forms of the latter species barely condense. In various gel systems, heterogeneity among histone band patterns between higher eukaryotes, American and African trypanosomes has also been shown. In *T. b. brucei*, H1 resolves as a fast migrating group of four variants and/or post-translational modifications, one of which is over expressed in the bloodstream form (Burri et al. 1994). All this indicates that trypanosomes display an alternative method of organising and processing the genetic information in their nucleus and several authors suggested that new possibilities of parasite control may arise from these observations (Hecker et al. 1995; Galanti et al. 1998).

E. Grüter · B. Betschart
University of Neuchâtel, Institute of Zoology,
Department of Parasitology, 2007 Neuchâtel, Switzerland
E-mail: bruno.betschart@zool.unine.ch
Tel.: +41-32-7183045
Fax: +41-32-7183001

In African trypanosomes, histone H1 has not yet been described at the genetic level. In the present paper, we describe the isolation and molecular characterisation of the gene encoding the H1 protein in *T. brucei*. We also show that the genes are organised in tandem repeats and that they may be separated into two clusters which could be implicated in differential H1 expression during the life cycle. The observed sequence divergence between African and American trypanosomes confirms the early separation of the *Salivaria* (Haag et al. 1998) and the gene heterogeneity among subspecies and strains shows a faint conservation of histone genes when examining protozoans.

Material and methods

Parasitic material and libraries

The λ gt11 cDNA library of *Trypanosoma brucei gambiense* (1257) prepared by Barnes et al. (1989) was a kind gift of T. Seebeck and an additional cDNA library of *T. b. brucei* (427) was provided by I. Roditi, both from the University of Bern. *T. b. gambiense* (STIB755) and *T. b. brucei* (Treu 927/4) were obtained from the Swiss Tropical Institute (Basel). Procyclic forms were cultured to a density of 10^7 /ml in SM medium supplemented with 10% heat inactivated fetal calf serum (Cunningham 1977). Bloodstream forms were propagated in CD1 mice (Charles River, Germany) by intraperitoneal inoculation with 10^5 trypanosomes. After 7 days, blood was harvested by heart puncture and trypanosomes were separated from blood cells by anion exchange chromatography through DEAE cellulose (Lanham 1970).

cDNA library screening

The λ gt11 cDNA grown in *Escherichia coli* strain Y1088 was screened by standard plaque lift procedures (after Stratagene Protocols, 1993). Three biotinylated oligonucleotides corresponding to parts of previously sequenced peptide fragments (Burri et al. 1995) aligned to an EST (T223) showing strong homology with H1 histones (El-Sayed et al. 1995) were used for hybridisation with a chemiluminescent (Biotin-Streptavidine) kit (TROPPIX). Probes were: TH1S1: 5'-AAGGCTGTCGCTAAG-3'; TH1S2: 5'-GCTAAGAAGGCTGCT-3'; TH1S3: 5'-GCACTGCTGCTCCC-3'. Washes were performed according to manufacturer's instructions. The selected clones were then amplified using standard forward and reverse λ gt11 primers and subcloned into Bluescript plasmid SK- for sequencing.

Subcloning and sequencing

cDNA inserts and all other PCR amplified fragments were subcloned into Bluescript SK- plasmid using the "Plasmid + T" method (Marchuk et al. 1991), except for the PCR products obtained with primer 5'-MAKTT/1 (see below) which were cloned into TOPO TA cloning kit (Invitrogen). Sequencing was carried out on a LICOR 4000 automated sequencer (MWG) using a Thermosequenase sequencing kit (Amersham) and both M13 universal or reverse dye-labelled primers (IRD800). Sequence analysis was performed with "DNA StriderTM 1.0.1" and alignments were made with ClustalW (Thompson et al. 1994). BLAST analyses were made at NCBI, EMBL and the EBI Parasite Genome blast server.

DNA and RNA manipulations

Genomic DNA was extracted by the standard phenol procedure (Sambrook et al. 1989) with a prolonged overnight proteinase K

digestion step. Specific isolation of H1 genes was done using four primers encompassing the different open reading frames (ORFs). Primers 5'-MNNTT (5'-CTTTATCGACTCCCCACAAG-3'), 5'-MAKAS (5'-CAATCTTATCAACTCGGAAG-3') and 5'-MAKTT/1 (5'-GAACATTTTCATAGGAAAGTAGAAAGG-3') are located upstream of the start codon of different H1 variants and primer H1orf3' (5'-CACTCAGGTAAACCTCATAGC-3') is situated in a conserved region downstream of the stop codon. A second primer corresponding to MAKTT variants was also used: 5'-MAKTT/2 (5'-GAAAGTAGAAAGGAAAATAAAATATGG-3') and a primer located on the 5'-coding sequence of MNNTT variants and oriented upstream allowed analyses of relative gene positioning: Walk5'-MNNTT (5'-GCCTTCACAGTAGCGGTTG-3'). The PCR amplifications were performed with the following cycling conditions: 94°C×2 min, 30 times: 94°C×30 s, Tm-3°C×30 s, 72°C×1 min, and a final elongation step at 72°C×5 min. Total RNA was extracted using TRIZOL Reagent (GIBCO BRL, Life Technologies) according to manufacturer's instructions. Theoretical secondary structures were highlighted with the GCG "Stem-loop" program (Devereux et al. 1984). Selective RT-PCR was performed according to standard procedure using an oligo-dT primer for reverse transcription (42°C). The second strand was amplified using primer TrypSL (5'-CGCTAT-TATTAGAACAGTTTCTG-3') corresponding to part of the spliced-leader and the conserved primer H1orf3'. Northern blots were performed after standard procedures (Ausubel et al. 1995). Blots were hybridised against specific biotinylated DNA probes corresponding to the different ORFs. Calibration was made against tubulin alpha, a kind gift from T. Seebeck from the University of Bern.

Results

Histone H1 gene isolation

Several cDNA clones from a λ gt11 library of *T. b. gambiense* (1257) were isolated using the three oligonucleotide probes TH1S1, TH1S2 and TH1S3 (see Material and methods). All isolated clones displayed a high level of sequence similarity with only one clone showing a different 5'-coding and non-coding sequence. The region downstream the stop codon was always highly conserved in all isolated clones (data not shown; GenBank accession numbers AJ272459-462).

Three primers based on the information obtained from screening were used in order to achieve the gene isolation by PCR from the genomic DNA of *T. b. brucei* (TREU927/4). Two PCR reactions associating primers 5'-MNNTT/H1orf3' or 5'-MAKAS/H1orf3' were performed (Fig. 1: lanes 1-4). In both cases, fragments of about 300-350 bp were amplified, the one integrating the 5'-MAKAS primer being slightly smaller and smeary, indicating that it probably contained a mixture of closely sized fragments. Bands ranging around 900 bp and 1,500 bp (Fig. 1, lanes 1, 3) were also observed. After subcloning and sequencing of the two 300-350 bp bands, several similar but different deduced ORFs could be grouped into four different size classes coding for proteins of respectively 80, 76, 71 and 61 amino acids (aa) (Fig. 2). The longest variant presents a specific 11 aa N-terminal end and the three smaller ones correspond to variants differing from each other only by some substitutions and/or deletions in their simple and repetitive sequence. The

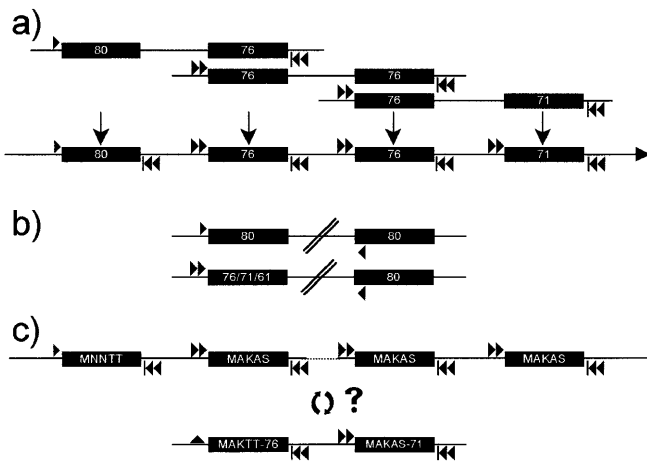


Fig. 3 a Schematic representation of the different sequenced H1 variants tandems (80, 76 and 71 aa) and hypothetical reconstruction on the baseline. b Representation of the two controls that gave no PCR amplification; double bars unsuccessful PCR amplification. c Schematic arrangement of the three different H1 variants groups; arrows show unknown relationship between the two sets of genes. single arrow right primer 5'-MNNTT; double arrow right primer 5'-MAKAS; bar with double arrow left primer H1orf3'; single arrow left primer Walk5'-MNNTT; arrow up primer 5'-MAKTT/1

performed. The PCR combined a primer on the spliced-leader Tryp-SL and the H1-specific primer H1orf3'. All the sequences obtained after subcloning exhibited the same splicing site, 27 bases upstream the ATG codon for MNNTT clones (data not shown; GenBank accession number AJ287604) and 29 bases for the others (Fig. 4; GenBank accession numbers AJ287605–606). However, alternative splicing cannot be excluded since only a few clones were analysed. Using this method, no spliced cDNA containing a MAKTT sequence could be obtained.

Fig. 4 Sequence representation of a tandem composed of an 80 aa variant followed by a 76 aa variant. Capital letters show the two ORFs; italicised letters in the intergenic sequence locate the predicted stem-loop (363–408); capital letters in italics show the polypyrimidine tract (561–654); grey boxes substitutions when compared to the intergenic-sequence separating other variants, above for 76–71 variants and below for 76–76 variants; arrow down trans-splicing site; singly underlined primer 5'-MNNTT; double underlined primer 5'-MAKAS; dotted underlined primer H1orf3'; open boxes potential splicing sites



This RT-PCR procedure does not allow the recovery of complete cDNAs. However, several *T. b. gambiense* cDNA clones obtained by screening were polyadenylated. The used sites were all closely located, 10–30 bases upstream of the polypyrimidine tract with one exception in which the polyadenylation site was located about 100 bases further upstream (data not shown).

When submitting the different IRs to computer analysis for theoretical stem-loop formation, which has been shown to be able to affect expression (Hehl et al. 1994), the longest and most stable formation involved the region of high variability. The stem-loop is only seen in the 3'UTR of MNNTT and MAKTT mRNAs and is composed of 15 stem-bases, the loop consisting almost exclusively of uracil (Fig. 4).

Northern blots comparing the global transcription rate of histone H1 genes during the two life-stages of the parasite showed no significant difference (Fig. 5a). In addition, the presence/absence of transcripts corresponding to the three subtypes of genes was analysed, but all three types were well amplified on RT products of both stages of the life-cycle (Fig. 5b). Note that primer MAKTT/1 (Fig. 5b, lanes g–i) gave no amplification, probably because it is located over the splicing site. A second primer, MAKTT/2, was then constructed with its 5'-end on the next GA dinucleotide. This primer resulted in good amplification and sets the splicing site for this type of genes 23 bases upstream from the start codon (Fig. 5b, lanes j–l).

Similarity searches

When submitting the H1 sequences to BLAST searches, the sequences presenting the highest similarity are, indeed, the different *T. cruzi* H1 variants. The lysine pairs

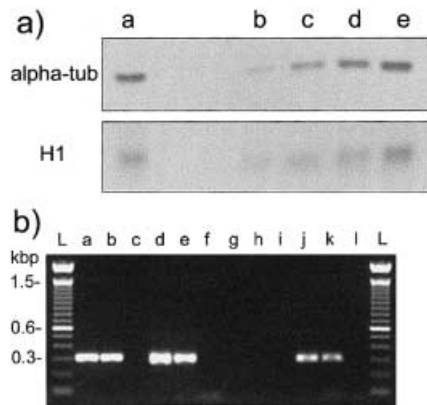


Fig. 5 a Global H1 transcription between the two parasitic life-stages by northern blot. *a* Bloodstream forms; *b-e* serial dilutions of procyclic forms RNA. *H1* Hybridisation with a mixture of four DNA probes corresponding to the four H1 size classes; *alpha-tub* calibration against an *a-tubulin* specific DNA probe. **b** Analysis of transcription of the three H1 gene subtypes in both stages of the life cycle. *L* 100 bp DNA ladder. *Lanes a, d, g, j* Amplification on procyclic RT-PCR product; *lanes b, e, h, k* amplification on bloodstream forms RT-PCR products; *lanes c, f, i, l* respective negative controls. Sets of primers are: 5'-MNNTT/H1orf3' (*lanes a-c*); 5'-MAKAS/H1orf3' (*lanes d-f*); 5'-MAKTT/1/H1orf3' (*lanes g-i*); 5'-MAKTT/2/H1orf3' (*lanes j-l*)

organisation is less regular and the variety of residues is greater in *T. cruzi* (Fig. 6a), but apart from this, size and general primary structure appear similar.

Interestingly, three other proteins showed yet a better score (Fig 6b). The first is a recently isolated H1 of *Leishmania brasiliensis* (unpublished, GenBank accession number AF131892) that exhibits a C-terminal region very similar to the one seen in African trypanosomes. The second is a histone H1 homologue from the prokaryote *Bordetella pertussis* (Scarlato et al. 1995). The central region of the latter contains the same repetitive pattern of two lysines followed by three non-polar and hydrophobic amino-acids (A, V, P or L). The third protein (data not shown) is referred to as a *T. b. brucei* microtubule associated protein and matches

perfectly the 3'-coding region of H1 clones but it is incomplete, lacking the 5' end (unpublished, GenBank accession number L41654).

Discussion

Primary structure and sequence motifs

Our results correlate well with previous studies on *T. b. brucei* in which four H1 variants and/or post-translational modifications have been described (Burri et al. 1993, 1994, 1995); Schlimme et al. 1993) as well as with the situation encountered in *T. cruzi* where a tandemly arranged gene family coding for histone H1 has been described (Åslund et al. 1994). This gene family is also formed by several very similar histones, the longest of which displays a different 20 aa long N-terminal region. Apart from these short special regions, the H1 genes of *T. brucei* and *T. cruzi* correspond only to the C-terminal tails of higher eucaryote H1, and completely lack the usual central globular domain and N-terminal tail. The simpler structure and small size appear to be a general feature of trypanosomatid H1 histones (Galanti et al. 1998). When compared to H1s of *T. cruzi*, the relatively simple aa composition and primary structure of African trypanosome H1 histones already give some interesting indications of their ability to undergo post-translational modification. Threonine and serine (only one residue in *T. b. brucei*, three to four in *T. cruzi*) exhibit a hydroxyl moiety allowing phosphorylation, which is thought to modulate the binding efficiency to chromatin by reducing the net positive charge of H1 (Van Holde 1989). In previous studies, H1s of *T. b. brucei* (Burri et al. 1995) and *T. cruzi* (Åslund et al. 1994) have been shown to be phosphorylated. However, the deduced sequences do not exhibit the well-defined "(S/T)-P-X-(K/R)" phosphorylation motif (Hill et al. 1990) present 1-3 times in *T. cruzi*. In addition, SPKK motifs have also been shown to be nucleic acid-binding units of proteins (Churchill and Suzuki 1989). Among

Fig. 6a, b Alignment of *T. b. brucei* H1 variant H1B76 with other proteins resulting from a BLAST search. **a** *Tcr T. cruzi* cDNA clone c8; grey boxes indicate substitutions of lysines by arginines found in *T. cruzi*; singly underlined indicates the less regular spacing of the lysine pairs. **b** *Bp* H1 homologue of the prokaryote *B. pertussis*; *Lbr* H1 of *L. brasiliensis*. * end of sequence; | matching positions

a)			
TbbH1-76B	MAKASAAPKKAVAKKAAPKKAAPKKAVAKKAAPKKAVAKKAAPKKAVAKKPLAKKVVAKK		
Tcr (c8)	MSDAAVPPKKASPKKAAKKASPKKAAARKTAAKKTAKKPAVRKPAAKKRAAPKKKPAAK		
TbbH1-76B	VAPKKVAGKKAACKA* 76		
Tcr (c8)	KAPKKAV-KKAPKKK* 74		
b)			
Bp	61..VAKKAVAKKAVAKKAVAKKAVAKKAVAKKAVAKKAPAKKAVAKKAVAKKAVAKKA		
TbbH1-76B	-----MAKASAAPKKAVAKKAAPKKAAPKKAVAKKAAPKKAVAKKAAPKKAVAKK		
Lbr	14..SNSPQRSPPSPKKAAVKKAACKAAAKKAAAPKKAAPKRAAPKRAAPKKAAPKKAACKRA		
Bp	VAKKAVAKKAVPKKAPAKKAAPKKPATPPSTAAAPGAKTALN..163 182		
TbbH1-76B	LAKKVAVKKVAPKKVAGKKAACKA* 76		
Lbr	-AKKSAPKKAVKKAVKAAKAVKKAACKAT* 102		

the *T. b. brucei* H1 proteins, only the MNNTT variants exhibit such a motif, at the end of the specific N-terminus, with a threonine instead of serine (Suzuki 1989). The unique serine situated at position 5 on MAKAS-76, -71 and -61 variants corresponds to other phosphorylation motifs, the serine being flanked by two hydrophobic residues and immediately preceded by a basic residue (lysine or arginine) (Hohmann 1983). The two threonines present in the recently isolated MAKTT variants do not resemble classic phosphorylation sites. The absence of multiple phosphorylation sites and/or DNA binding motifs in *T. brucei* H1s could explain the differences seen in chromatin compaction when compared to *T. cruzi* (Hecker et al. 1994).

H1 gene heterogeneity

A certain heterogeneity within the three smallest polypeptide classes was found and, in addition, some nucleotide substitutions did not give rise to aa changes. In sequences coding for MNNTT-80 variants, the only substitution concerned the codon usage for the lysine at position 64 which was either “aaa” or “aag” with an equal distribution among the six clones that were analysed.

Using the same PCR isolation procedure on genomic DNA from *T. b. gambiense* (STIB755), at least seven size classes of H1 genes coding for proteins of 61–91 aa were obtained. The N-terminal domain found on MNNTT-80 variants of *T. b. brucei* was present on two intermediate variants of 85 and 75 aa with the alanine at position 6 being substituted by aspartic acid (Grueter 2000; GenBank accession numbers AJ287591–597).

The isolation of four H1 size classes in *T. b. brucei* and seven in *T. b. gambiense*, with each class displaying additional sequence heterogeneity as well as the presence of one type of gene coding for a short extra N-terminal motif and another type which appears distant from the previous ones and exhibits a threonine pair, denote a very complex situation. In addition, a cDNA clone coding for H1 from a different *T. b. brucei* strain (427) which displayed a new intermediate size of 66 aa was recently isolated (GenBank accession number AJ287608). Even if it cannot be excluded that this variant was missed in strain TREU 927/4 during isolation procedures, this new variant, and the additional fact that it exhibits a number of other nucleotide substitutions in its non-coding sequence, tends to demonstrate heterogeneity even at the strain level. Species and strain heterogeneity have also been reported in *Leishmania* (Belli et al. 1999).

Genome organisation

All isolated H1 genes are tandemly organised. However, the absence of experimental evidence that MAKTT genes are closely linked to the other variant ORFs

strongly suggests that two H1 clusters may be present. The existence of two MNNTT genes differing by only one nucleotide as well as two MAKTT-76 variants adds to the complexity of this gene family but these may also represent allelic forms.

The T223 EST (see Material and methods) only hybridises to chromosome XI (see website of African Trypanosome Genome Project, EBI) and therefore all H1 genes or clusters should be grouped as has been shown for histone H2A genes in *T. cruzi* (Puerta et al. 1994). The same source of information also shows that core histone genes are located on different chromosomes. This was also described for *T. cruzi* (Åslund et al. 1994) and represents a unique type of histone gene organisation among eukaryotes.

The over-expressed variant

Among the four *T. b. brucei* H1 variants, the one which is over-expressed in bloodstream forms (Burri et al. 1994) could not be identified according to previous aa composition analyses (Burri et al. 1993). Even if the gross repartition of the different aa involved are well verified, these former results do not reflect the real composition of the gene products. The same kind of divergence was seen in *T. cruzi* (Toro et al. 1993; Åslund et al. 1994) and is probably due to the heterogeneity of this gene family. However, the peptide fragment H1.3 sequenced by Burri et al. (1995), which is related to the over-expressed variant, best aligns to the MAKAS-76, -71 and -61 variants. When considering the separation according to the molecular weight in 2D gels (Burri et al. 1994), the over-expressed variant appears to be the second in size, i.e. the 76 aa variant, and in all isolation approaches the 76 aa variants were always the best represented. In addition, the recent isolation of the MAKTT-76 H1 variant further indicates that it is not the MNNTT variant which presents several specific features (head position, different 5'-sequence, 3'-UTR stem-loop) that is over-expressed.

The fact that MAKTT variants were never isolated, either from cDNA libraries or by selective RT-PCR which was always performed on procyclic forms, may signify that MAKTT genes are differentially regulated over the life-cycle. However, MAKTT transcripts were amplified in both stages, suggesting that regulation may then occur post-transcriptionally (Graham 1995).

Splitting of H1 genes into two clusters may allow additional modulation of relative histone expression levels. In line with this hypothesis, enhanced chromatin compaction was seen when increasing ratios of H1 to core histones were applied in reconstitution experiments on *T. cruzi* chromatin (Schlimme et al. 1995). Differences in chromatin compaction and stability between stages may therefore simply depend on increased H1 synthesis rather than on expression of H1 displaying different biochemical properties.

Functionality

The surprising assignation of the perfectly aligned sequence to a microtubule associated protein as found by BLAST searches could be due to the microtubule purification procedure which may have allowed co-extraction of H1. Another hypothesis could be that histone H1 has still unclear functions in trypanosomes, since chromatin condensation does not occur in a significant way. In agreement with this hypothesis is the finding that H1 of sea urchin sperm cells was shown to stabilise microtubules, and that antibodies raised against this protein also recognised organelles like the flagellum or cilia in different lower eukaryotes (Multigner et al. 1992). Despite this, preliminary immunolocalisation experiments with a specific anti-*T. b. brucei* H1 antibody showed no extranuclear signal (Grueter 2000).

The fine nuclear localisation of these short H1s, their interactions with other structures and their precise expression timing should now be investigated in greater detail in order to achieve a more precise comprehension of histone H1 functionality in trypanosomes.

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