Sequence differences between histones of procyclic *Trypanosoma brucei brucei* and higher eukaryotes

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SUMMARY

Four histones, a, b, c, d from procyclic *Trypanosoma brucei brucei*, which show similarities with the amino acid composition of the core histones H3, H2A, H2B and H4, were isolated and cleaved with Endoproteinase Glu-C. The fragments were separated by FPLC reversed phase chromatography and a subset of the fragments (a5, a9, b6, c8, d3, d9, d11) was subjected to sequence analysis. A 54–71 % identity was found in the sequences of the fragment c8 and the C-terminal half of H2B and of three fragments of protein d covering the N-terminal half as well as the C-terminal region of H4. The amino acid sequence of the fragment a9 showed a 57 and 54 % identity with H3 sequences of *Saccharomyces cerevisiae* and *Xenopus laevis*. Neither the a5 nor the b6 sequence could be aligned with histone sequences of other eukaryotes. The significant differences of 21–48 % between the *T. b. brucei* histone sequences and those of calf thymus histones, which are more pronounced than the differences of *Tetrahymena pyriformis* and the higher eukaryote, resulted partially from replacements of amino acids with different properties and indicate specific patterns of histone–histone and/or histone–DNA contact sites in the nucleosome of *T. b. brucei*. These differences, together with the lack of a functional histone H1, may be sufficient to explain the lack of a salt-dependent formation of the nucleosome filament into the 30 nm fibre, which reflects alternative methods of organizing and processing the genetic information in the nucleus of the protozoan parasite and which may be of chemotherapeutic significance.

Key words: *Trypanosoma brucei brucei*, histone, amino acid sequence, histone variation, histone phylogeny.

INTRODUCTION

The nucleosome core particle, the basic subunit of chromatin of eukaryotic cells, is composed of two molecules of histone H2A, H2B, H3, H4, and a stretch of 146 bp of DNA (Van Holde *et al.* 1975; McGhee & Felsenfeld, 1980). Core histones have been generally conserved during evolution (DeLange *et al.* 1969*a*; Isenberg, 1979; Van Holde, 1989) in particular the portions of the histone molecules which constitute the central globular core. This implies that histone–histone interaction is of a conservative nature (Martinson *et al.* 1979). However, relatively large amino acid sequence differences may exist between the core histones of lower and higher eukaryotes. The largest differences of 13–37 %, between any core histones hitherto described, are those between *Tetrahymena pyriformis* and human spleen or calf thymus histones (Nomoto, Hayashi & Iwai, 1982; Fusauchi & Iwai, 1983; Hayashi *et al.* 1984*a*; Hayashi, Nomoto & Iwai, 1984*b*; Kasai, Hayashi & Iwai, 1986). It is postulated that the degree of diversity of core histones among the eukaryotes did increase with the capacity to undergo post-translational modifications which occur mainly in the basic, N-terminal regions (Isenberg, 1979). These regions were proposed to be involved in the modulation of fibre-solenoid transitions rather than in the stabilization of the nucleosome itself (McGhee & Felsenfeld, 1980; Allan *et al.* 1982). Four histones a, b, c and d are involved in the organization of the nuclear chromatin of procyclic *Trypanosoma brucei brucei* (Hecker & Gander, 1985; Hecker *et al.* 1989; Bender *et al.* 1991). Similarities in the amino acid composition were found for the histones a and H3, b and H2A, c and H2B, d and H4, but these histones could be distinguished from those of higher eukaryotes by differences in charge and/or size. Proteins a and d showed a lower hydrophobicity than H3 and H4 (Bender *et al.* 1991).

The chromatin organization of *T. b. brucei* differs from that of higher eukaryotes. Protein–DNA interactions are less stable than in rat liver chromatin (Hecker *et al.* 1989). The interactions between the histones a and d with the DNA were abolished at significantly lower ionic strength as compared to those of H3 and H4 with the DNA in the chromatin of higher eukaryotes (Bender *et al.* manuscript submitted). In addition, no functional histone H1 could be demonstrated in *T. b. brucei* chromatin, nucleosomes were arranged and spaced irregularly and no salt-induced formation of the chromatin filaments into the typical 30 nm fibre took place (Hecker & Gander, 1985; Hecker *et al.* 1989).

It was the aim of the present investigation, to

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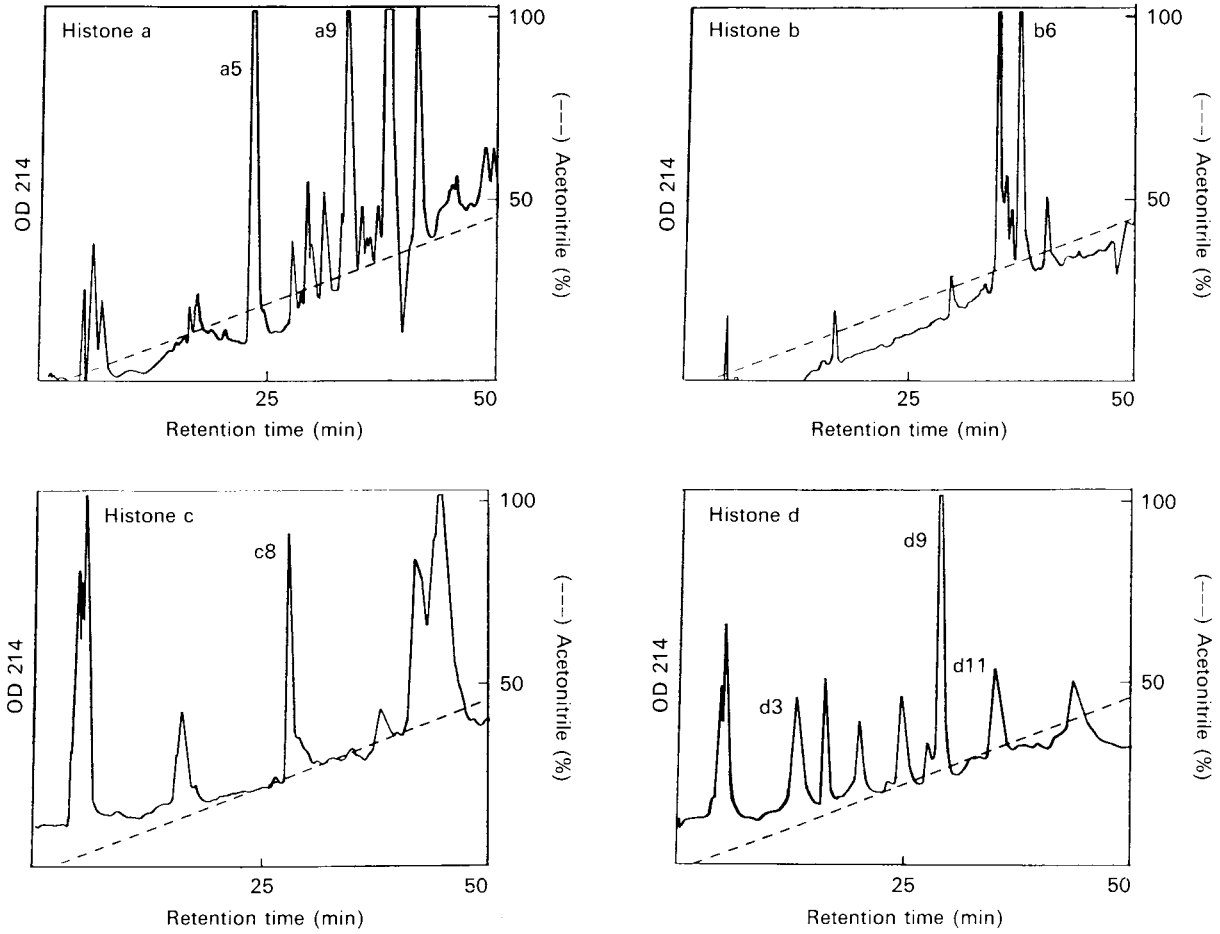

Fig. 1. Separation of peptide fragments generated by cleavage of procyclic *Trypanosoma brucei brucei* histones a, b, c, d with Endoproteinase Glu-C. Reversed phase FPLC on a C1/C8 column (Pharmacia, ProRPC 5/10) using a linear acetonitrile gradient. Solution A, 0·1 % TFA in water. Solution B, 0·1 % TFA in acetonitrile. Peaks used for sequence analysis are marked.

a5: Thr–Ala–Arg–Thr–Lys–Lys–Thr–Ile–Thr–Ser–Lys–Lys–Ser–Lys–Lys

a9: Ile–Thr/Leu–Gln–Phe–Gln–Arg–Ser–Thr–Asp–Leu–Leu–Leu–Gln Lys–Ala

b6: Leu–Ser–Val–Lys–Ala–Ala–Ala–Gln–Gln–Thr–Lys–Lys–Thr–Lys–Arg–Leu–Thr–Pro–Arg–Thr

c8: Leu–Gln–Thr–Ala–Val–Arg–Leu–Val–Leu–Pro–Ala–Asp–Leu–Ala

d9.1: Tyr–Ser–Arg–Lys–Lys–Thr–Val–Thr–Ala–Val–Asp–Val–Val–Asn–Ala–Leu–Arg–Lys–Arg–Gly–Lys–Ile–Leu–Tyr–Gly–Tyr–Ala

d9.2: Ile–(Ser)–Gln–Phe–Gln–(Arg)–Ser–Gly–Asp–Leu–Arg/Leu–(Leu)–Gln–Lys–(Arg)–Pro–Phe–Gln–(Arg)–Leu

d11: Asn–Val–Xaa–Gly–Ile–Thr–Arg–Gly–Ser–Ile–Arg–Arg–Leu–Ala–(Arg)–Xaa–Gly–Xaa–Val

d3.1: Xaa–Lys–Gly–Xaa–Lys–Ser–Gly–Glu–Ala–Lys–Gly–Ser–Gln–(Lys)–Arg

d3.2: Lys–Gly–Xaa–Lys–Ser–Gly–Glu–Ala–Lys–Gly–Ser–Gln–(Lys)–Arg

d3.3: Ala–(Lys)–(Gly)–Ser–Gln–(Lys)–Arg–Gln–(Arg)–Xaa–Val–Leu–Arg

Fig. 2. Amino acid sequences of procyclic *Trypanosoma brucei brucei* histone fragments a5, a9, b6, c8, d9.1., d9.2., d11, d3.1., d3.2., d3.3.. Xaa, unidentified residue; /, ambiguity between two residues; (...), most probable residue.

analyse the amino acid sequence of fragments of the histones of *T. b. brucei* in order to establish their identity with those of other eukaryotes on the basis of their primary structure. Differences between *T. b. brucei* histones and those of higher eukaryotes should contribute to the better understanding of histone evolution as well as to the identification of potential target sites for new chemotherapeutic agents for African trypanosomiases.

MATERIALS AND METHODS

Trypanosoma brucei brucei procyclic culture forms were cultivated in SDM 79 medium (Brun & Schoenenberger, 1979).

Preparation of nuclei

Nuclei were isolated from $1{\cdot}5–3{\cdot}5 \times 10^{10}$ exponentially growing procyclic *T. b. brucei* (Hecker *et al.* 1989; Shapiro & Doxsey, 1982).

Production of soluble chromatin

Isolated nuclei were digested at 30 °C for 50 sec with micrococcal nuclease (Sigma, N-3755, 0·2U/20 A260), centrifuged, and the chromatin solubilized by nuclear lysis in a low-salt buffer. Insoluble material was removed by centrifugation (Hecker & Gander, 1985; Thoma, Koller & Klug, 1979).

Extraction of histones from soluble chromatin

Histones were extracted according to the method of Kurochkina & Kolomijtseva (1989) from the lyophilized, soluble chromatin with 0·2 M H_2SO_4. Insoluble material was removed by centrifugation (11500 ***g***, 15 min). Histones were precipitated with 4·5 vol. ethanol at −100 °C, centrifuged, the pellet washed with 80 % ethanol and vacuum dried.

Purification of histones

Extacted histones were separated by FPLC reversed phase chromatography with acetonitrile gradients as described previously (Bender *et al.* 1991).

Cleavage of histones with Endoproteinase Glu-C

Histones were digested with Endoproteinase Glu-C from *Staphylococcus aureus* (Boehringer, 1/20 w/w) in 25 mM ammonium carbonate buffer (pH 7·8) for 2 h at 25 °C following the methods of Ohe, Hayashi & Iwai (1989) and Drapeau (1977).

Isolation of the histone fragments

Fragments were isolated in a FPLC-system connected to a reversed phase C1/C8 column (Pharmacia, ProRPC 5/10). Histone solutions were taken up with 0·1 % TFA to 200–500 µl and chromatographed at a constant flow of 0·5 ml/min, using a gradient of solvent A with 0·1 % TFA in water and solvent B with 0·1 % TFA in acetonitrile. The concentration of B was increased at a rate of 1 %/min. The eluted histone fragments were monitored at 214 nm. The peak fractions were collected separately and lyophilized.

Sequence analysis

N-terminal amino acid sequence analysis was performed by automated Edman degradation with a pulsed-liquid-phase sequenator from Applied Biosystems (model 477A, Foster City, Ca, USA). The released amino acids were analysed on-line according to Applied Biosystems (Schaller *et al.* 1991).

Sequence comparison

To assess the degree of similarity, amino acid sequences of trypanosome histone fragments were compared (Lipman & Pearson, 1985) to reference sequences by using the FASTA program and the NBRF data bank (March 1991; 26651 sequences).

RESULTS

The purified histones a, b, c, d (Bender *et al.* 1991) were cleaved with Endoproteinase Glu-C, and the resulting fragments were separated by FPLC reversed phase chromatography in 0–45 % acetonitrile gradients (Fig. 1). The amino acid sequences of 10 fragments of the 4 histones were analysed (Fig. 2). The fragments differed in size in having between 13 and 27 amino acids. Five out of the 7 peak fractions contained single peptides whereas peaks 3 and 9 of histone d were mixtures of several peptides in the ratios of 5:1:1 (d3.1., d3.2. and d3.3.) and 5:1 (d9.1. and d9.2.).

The degree of similarity between the amino acid sequences of the histones of procyclic *T. b. brucei* and histones of other eukaryotes was determined by sequence comparison using the NBRF protein data bank (Table 1). The comparison, of the sequences of the fragments d9.1., d9.2. and d11 with the ones in the library, scored within the 20 (of c8 within 33) most similar sequences, exclusively histones. H2B sequences were scored for c8, H3 for d9.2. and H4 for d9.1. and d11. Only a few scores for histones were found for the sequences of the other fragments (a5, a9, b6, d3.1., d3.2. and d3.3.). It should be mentioned that the sequence of fragment a5 showed a score with 5 H1 sequences from human, trout and *Caenorhabditis elegans* (MacLeod, Wong & Dixon, 1977; Mezquita *et al.* 1985; Doenecke & Tonjes, 1986; Vanfleteren, van Bun & van Beeumen, 1988; Sanicola *et al.* 1990) as well as two H5 sequences from goose and chicken (Yaguchi, Roy & Seligy, 1979; Krieg *et al.* 1983) among the 20 most similar sequences.

A high degree of sequence identity (64–71 %) was

Table 1. Comparison of procyclic *Trypanosoma brucei brucei* histone sequences c8, a9, d9.2., d9.1. and d11 with the NBRF sequence data bank

(March 1991; 26 651 sequences available.)

Fragments	Similar histones	Similar histone regions*	Identity (%)†
c8	H2B	90–103 (12)[c1]	64–71
		91–104 (18)	71
		94–107 (15)	71
		95–108 (9)	71
a9	H3	51–65 (5)[c2]	57
		81–95 (5)	54
d9.2.	H3	51–69 (5)	55
		51–70 (80)	60–65
		52–71 (10)	60
d9.1.	H4	75–101 (82)	54–58
		76–102 (18)	58
d11	H4	25–43 (80)	58–63
		26–44 (20)	63

* Numbers represent positions of amino acid residues in eukaryotic histones. [c1,c2] numbers in parenthesis indicate percentage of similar histone regions within the 33 (c1) or 20 (c2) best scores.

† Percentage identity between *T. b. brucei* sequences and histone sequences of other eukaryotes.

found for fragment c8 and the C-terminal half of histones H2B. Fragment d9.1. showed a high percentage of identity with the C-terminal portion and d11 with the N-terminal half of H4 histones. In the two scores of fragment a9 a sequence identity of 54–57% was found with H3 histones of *Xenopus laevis* and *Saccharomyces cerevisiae* (Moorman *et al.* 1981; Brandt & von Holt, 1982). Surprisingly, d9.2. showed a 55–65% identity with the central region of H3 as well (Table 1). Start and end positions of the most similar regions of corresponding histones found for d9.1., d9.2. and d11 differed in only one or two residues. Regions of H2B histones showing similarities to peptide c8 were somewhat more variable and 54% of the scored histone regions were located between residues 90–108.

DISCUSSION

The amino acid composition and the electrophoretic mobilities in three different gel systems of the four proteins a, b, c, d isolated from nuclear chromatin of procyclic *T. b. brucei* strongly indicated their core histone nature (Bender *et al.* 1991). Similarities of a, b, c and d with the core histones H3, H2A, H2B and *H4 of higher eukaryotes were found.*

On the basis of the relatively high number of glutamoyl residues per protein molecule, Endoproteinase Glu-C was used to produce fragments of medium size. The amino acid sequences, described in the present report, of some of these fragments of three proteins showed high scores and a high percentage of sequence identity with histones, which allowed the identification of protein a as H3, c as H2B and d as H4.

The amino acid composition as well as the electrophoretic behaviour of histone b indicated its relationship to H2A histones (Bender *et al.* 1991). The fragment b6 can, on the basis of the sequence comparison, not be identified as a H2A histone, which suggests that this fragment represents a region of histone b not well conserved during evolution, as it is known for the lysine- and arginine-rich terminal domain of H2A (Isenberg, 1979; Van Holde, 1989). It cannot be ruled out, however, that this fragment could be derived from a contaminating protein.

Fragment d3.1., representing 70% of fraction d3, can be extended by the 6 residues of d3.3. (positions 8–13) to yield the sequence Xaa-Lys-Gly-Xaa-Lys-Ser-Gly-Glu-Ala-Lys-Gly-Ser-Gln-(Lys)-Arg-Gln-(Arg)-Xaa-Val-Leu-Arg- (d3). The comparison of the combined sequence with the protein library yielded a significant similarity to the N-terminal region of H4 histones (positions 4(5)–23(24)) with 55–60% identity. The sequence of positions 1–7 in peptide d3.3., present in relatively small amounts in fraction d3, corresponded to the position 9–15 in peptide d3.1.. It is possible that the bond between glutamic acid and alanine at positions 8 and 9 of peptide d3.1. was partially cleaved.

Sequences d3.2. and d3.1. were identical, with the exception of their N-terminal amino acid, suggesting the fragment d3.2. was generated from d3.1. by unspecific cleavage between Xaa (1) and Lys (2). The unidentified residues in sequence d3 (in positions 1 and 4) eluted as peaks between the positions of tyrosine and proline and between those of histidine and alanine. These two residues of the sequence d3 correspond to the N-terminal region of H4 if the two sequences are aligned (Fig. 4). Amino acids such as serine, which is often found to be acetylated, and/or lysine which may be acetylated or methylated are found in the N-terminal region (residues 1–20) of H4 histones. It can therefore be assumed that Xaa (1) and Xaa (4) correspond to such modified amino acids (for a review, see Van Holde, 1989).

The 14 N-terminal residues of fragment d9.2., which represented a subfraction (17%) of d9, corresponded to the sequence a9 of histone a. The sequence differed only in the positions 2, 8 and 15. Since both fractions a9 and d9 eluted at similar acetonitrile concentrations of about 30% and since protein d preparations occasionally were contaminated with small amounts of protein a, it is reasonable to suggest that peptide d9.2. is a fragment of protein a.

All segments of the histones of eukaryotes that showed similarity to the *T. b. brucei* sequences a9 (resp. d9.2.), c8, d9.1., and the C-terminal part of d11 are within the globular, relatively hydrophobic,

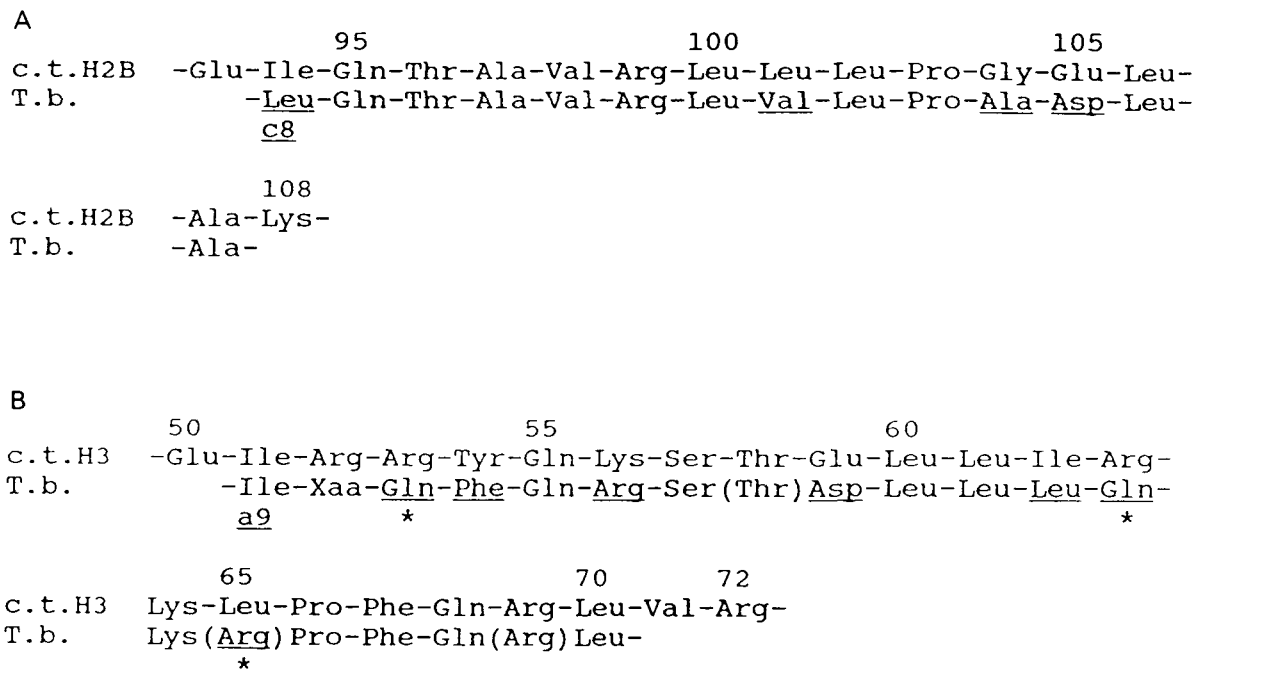

Fig. 3. Comparison of c8 and a9 histone fragments of procyclic *Trypanosoma brucei brucei* (T.b.) with (A) calf thymus (c.t.) histone H2B (Iwai, Hayashi & Ishikawa, 1972) and (B) H3 (DeLange, Hooper & Smith, 1973). Sequences were aligned to obtain maximum similarity. Xaa, unidentified residue; (...), most probable residue; —, amino acid substitution; * , replacement of residues with different properties.

region of the molecules. These are involved in the histone-histone interactions in the core particle of higher eukaryotes (McGhee & Felsenfeld, 1980). Such interactions have been proposed to exist between residues 37–114 of H2B and histone H2A (Moss *et al.* 1976), the C-terminal half of H2B and H4 (Martinson *et al.* 1979), and residues 42–120 in histone H3 and 42–102 in histone H4 (Böhm *et al.* 1977). Similarities between these histone regions of higher eukaryotes and the *T. b. brucei* sequences suggest contact sites between histones a and d, as well as c and d, a finding which is in agreement with the evolutionary conserved pattern of histone–histone binding (Isenberg, 1979; Martinson & True, 1979).

In spite of these similarities significant differences were found when the sequences were compared to calf thymus histones and aligned to obtain maximum similarity. Sequences c8 and H2B (Fig. 3) differed in the residues 94–107 to a degree of 29 % through substitutions between similar amino acids (Dayhoff, 1972; Schulz & Schirmer, 1984). A9 and H3 (residues 51–70, Fig. 3) were different at 7 positions (35 % difference) with 3 differences resembling substitutions not strictly conservative (Dayhoff, 1972; Schulz & Schirmer, 1984).

The successful alignment of the *T. b. brucei* sequences d3, d11, d9 with calf thymus histone H4 in the positions 4–23, 25–43 and 75–101 were different at 22 residues corresponding to a 33 % difference (Fig. 4): 5 of them were in the N-terminal region (24 % difference), 4 in the residue 25–43 (21 % difference), and 13 in the C-terminal region (48 % difference). Most substitutions were between similar amino acids, whereas 6 differences in the N- and C-terminal portions were substitutions between amino acids with different properties.

The amino acid N-terminally located to Leu (1), Ile (1) and Tyr (1) of the three peptides c8, a9 (Fig. 3) and d9 (Fig. 4) are probably glutamic acid as deduced from the specificity of Endoproteinase Glu-C and the occurrence of glutamic acid at positions 93, 50 and 74 in the corresponding calf thymus sequences.

The amino acid replacement of glutamic acid with leucine at position 8, of serine with glycine at position 12 of *T. b. brucei* d3 versus calf H4, and the presence of serine at position 6 of d3, are in addition to the Xaa (1 and 4) sites for possible post-translational histone modifications such as methylation (DeLange *et al.* 1969*a*; DeLange & Smith, 1971; DeLange, Hooper & Smith, 1973), ADP-ribosylation (Hayashi & Ueda, 1977), phosphorylation (Jackson *et al.* 1976; Ajiro & Nishimoto, 1985; Fusauchi & Iwai, 1984), and acetylation (Doenecke & Gallwitz, 1982).

The differences between core histone sequences of calf thymus and *Tetrahymena pyriformis* (ciliated protozoan) are the largest sequence differences of histones described in eukaryotes (Kasai *et al.* 1986; Nomoto *et al.* 1982; Fusauchi & Iwai, 1983; Hayashi *et al.* 1984*a*, *b*). A difference of 14 % exists between

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          1                5          8       9  10
c.t.H4  Ser-Gly-Arg-Gly-Lys-Gly-Gly-Lys-----Gly-Leu-Gly-Lys-
T.b.                -Xaa-Lys-Gly-Xaa-Lys-Ser-Gly-Glu-Ala-Lys-
                   d3                              *

                                      20                  25
c.t.H4  Gly-Gly-Ala-Lys-Arg-His-Arg-Lys-Val-Leu-Arg-Asp-Asn-Ile-
T.b.    Gly-Ser-Gln(Lys)Arg-Gln(Arg)Xaa-Val-Leu-Arg-    -Asn-Val-
                          *                             d11

                    30                  35                  40
c.t.H4  Gln-Gly-Ile-Thr-Lys-Pro-Ala-Ile-Arg-Arg-Leu-Ala-Arg-Arg-
T.b.    Xaa-Gly-Ile-Thr-Arg-Gly-Ser-Ile-Arg-Arg-Leu-Ala(Arg)Xaa-

                    44          75                  80
c.t.H4  Gly-Gly-Val-Lys-...-Glu-His-Ala-Lys-Arg-Lys-Thr-Val-Thr-
T.b.    Gly-Xaa-Val-            Tyr-Ser-Arg-Lys-Lys-Thr-Val-Thr-
                                 *
                                d9

              85                  90                  95
c.t.H4  Ala-Met-Asp-Val-Val-Tyr-Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-
T.b.    Ala-Val-Asp-Val-Val-Asn-Ala-Leu-Arg-Lys-Arg-Gly-Lys-Ile-
                          *                   *           *

                    100     102
c.t.H4  Leu-Tyr-Gly-Phe-Gly-Gly
T.b.    Leu-Tyr-Gly-Tyr-Ala-
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Fig. 4. Comparison of amino acid sequences of histone d fragments (d3, d11, d9) of procyclic *Trypanosoma brucei brucei* (T.b.) with the sequence of calf thymus (c.t.) H4 (DeLange *et al.* 1969*b*). Sequences were aligned to obtain maximum similarity; the gap in the sequence of H4 between residues Lys (8) and Gly (9) was introduced to maximize similarity. Xaa, unidentified residue; (...), most probable residue; —, amino acid substitution; * , replacement of residues with different properties.

the residues 94–107 of calf thymus and the protozoan H2B; and of 10–15 % between the residues 51–70 of H3. Differences of 14–24 % (residues 4–23), 5 % (residues 25–43) and 4 % (residues 75–101) existed between calf thymus and the corresponding *T. pyriformis* H4 sequences. The sequence differences of 21–48 % between procyclic *T. b. brucei* and calf thymus histones were more pronounced than those beween *T. pyriformis* and calf thymus. These results are in agreement with the phylogenetic theory, that the separation of protozoa from higher eukaryotes occurred long before the one of animals from plants and fungi (Nomoto *et al.* 1982; Fusauchi & Iwai, 1983; Hayashi *et al.* 1984*a*, *b*), since sequence differences within corresponding core histones are small between the three phyla (Van Holde, 1989; Hayashi *et al.* 1984*a*, *b*).

The described substitutions of amino acids in procyclic *T. b. brucei* histone sequences may influence protein conformation and histone-histone as well as histone-DNA interactions within the nucleosome core of the protozoan parasite. The previous observation that nucleosome filaments of *T. b. brucei* were more easily destabilized under experimental conditions as compared to rat liver chromatin (Hecker *et al.* 1989) support this hypothesis. In addition, preliminary data indicate that histones a and d of procyclic *T. b. brucei* were more weakly bound to the DNA than H3 and H4 in the chromatin of higher eukaryotes (Burton *et al.* 1978), since they were released at 1 M NaCl (Bender *et al.* manuscript submitted). All these data indicate differences in the nucleosome structure, which are involved in the different compaction pattern of the chromatin of *T. b. brucei* (Hecker & Gander, 1985; Bender *et al.* manuscript submitted).

As a vaccine is still not available, chemotherapy is considered the most effective measure to improve the control of trypanosomiasis (Doyle *et al.* 1984; Mehlhorn 1988). Differences found between the histones of *T. b. brucei* and those of higher eukaryotes encourage the identification of new targets for the action of trypanocidal drugs. In addition to drugs directed against glycolytic enzymes, polyamines or the purine metabolism (Clement, 1989), putative chemotherapeutic agents should be designed which

bind selectively to the parasites' histones. They could interfere with the nucleosome assembly as well as with the transport of histones from the cytoplasm into the nucleus (Van Holde, 1989), thereby allowing a non-toxic chemotherapy of the host.

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