Trypanosoma brucei brucei: differences in the nuclear chromatin of bloodstream forms and procyclic culture forms

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SUMMARY

Nucleosome filaments of two stages of the life-cycle of *Trypanosoma brucei brucei*, namely bloodstream forms and procyclic culture forms, were investigated by electron microscopy. Chromatin of bloodstream forms showed a salt-dependent condensation. The level of condensation was higher than that shown by chromatin from procyclic culture forms, but 30 nm fibres as formed in rat liver chromatin preparations were not found. Analysis of histones provided new evidence for the existence of H1-like proteins, which comigrated in the region of the core histones in SDS–PAGE and in front of the core histones in Triton acid urea gels. Differences were found between the H1-like proteins of the two trypanosome stages as well as between the core histones in their amount, number of bands and banding pattern. It can be concluded that *T. b. brucei* contains a full set of histones, including H1-like proteins, and that the poor condensation of its chromatin is not due to the absence of H1, but most probably due to histone–DNA interaction being weak. It is obvious that structural and functional differences of the chromatin exist not only between *T. b. brucei* and higher eukaryotes, but also between various stages of the life-cycle of the parasite. It is therefore not adequate to investigate the chromatin only of the procyclic culture forms as a model for all stages of the life-cycle of *T. b. brucei*.

Key words: *Trypanosoma brucei brucei*, chromatin, histones, lower eukaryotes, bloodstream forms, procyclic culture forms.

INTRODUCTION

Trypanosomes, the protozoan parasites of man and animals, have various nuclear features different from those of higher eukaryotes. No condensed chromosomes can be visualized during nuclear division in trypanosomes (Vickerman & Preston, 1970), and the compaction of the chromatin in the nucleus is distinctly less pronounced as compared to the chromatin of rat liver nuclei (Hecker & Gander, 1985).

The chromatin of *T. b. brucei* and of *T. cruzi* procyclic culture forms was shown to be organized in a nucleosome filament-like form. However, the nucleosomes were spaced irregularly and no condensation into a typical 30 nm fibre took place under experimental conditions. The nuclear chromatin was digested rapidly by micrococcal nuclease, and the interactions between DNA and proteins were relatively weak and were easily destabilized under experimental conditions, which are normally used for the isolation of chromatin from higher eukaryotes (Hecker & Gander, 1985; Hecker *et al.* 1989; Bender, Betschart & Hecker, 1992*c*). In addition, no histone H1 could be demonstrated (Hecker & Gander, 1985; Hecker *et al.* 1989). Other workers also postulated that H1 was absent (Bender *et al.* 1992*a*, *b*, *c*, *d*). However, these results are controversial. Recent studies demonstrated H1-like proteins in *T. cruzi* and *Crithidia fasciculata* (Toro & Galanti, 1988, 1990; Duschak & Cazzulo, 1990). These histones showed biochemical properties similar to those of histones from *Tetrahymena fasciculata* (Johmann & Gorovsky, 1976).

On the basis of the migration pattern in various gel systems and amino acid sequence analysis, Bender *et al.* (1992*a*, *b*) were able to show that the four core histones of *T. b. brucei* procyclic culture forms, namely a, b, c, d are the counterparts of H3, H2B, H2A and H4 of higher eukaryotes. The core histones of higher eukaryotes are among the most conserved proteins known. Among the histones, H4 and H3 are the best conserved, followed by H2A, H2B and H1 (van Holde, 1989). Biochemical differences between the core histones of *T. b. brucei* procyclic culture forms and the core histones of higher eukaryotes were found by Bender *et al.* (1992*a*, *b*). Differences of about 35% exist in the amino acid sequence of the N- and C-terminal regions of the histone H4 of calf thymus and trypanosomes (Bender *et al.* 1992*b*; Toro *et al.* 1992).

All previous investigations have been carried out with *T. b. brucei* procyclic culture forms which can be cultivated to a density of 3×10^7/ml (Brun & Schönenberger, 1979). The *in vitro* cultivation of bloodstream forms, the parasitic stage affecting mammals, is possible only to a density of 2×10^6/ml (Hamm *et al.* 1990). Therefore studies on the

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chromatin of this stage depend on its propagation in laboratory animals. It was not known, whether the differences found between the chromatin of procyclic culture forms and that of higher eukaryotes would also be found in bloodstream forms.

In the present study, the chromatin of procyclic culture forms and bloodstream forms of *T. b. brucei* was compared with rat liver chromatin, using a variety of new separation techniques and staining methods. Also electron microscopy (EM) and enzymic digestion were used to find out whether there were differences between the chromatin of the two forms, and between that of bloodstream forms and higher eukaryotes. The validity of taking the chromatin of procyclic culture forms as a model for all the stages of the life-cycle of *T. b. brucei* was evaluated.

MATERIALS AND METHODS

Livers were from rats from the SIV IVANOVAS strain. *Trypanosoma b. brucei* STIB 345 AB procyclic culture forms, cultivated in SDM 79 medium containing 10% heat-inactivated foetal bovine serum, 10 μg/ml haemin and 10 μg/ml gentamicin at 27 °C were used (Brun & Schönenberger, 1979). Bloodstream forms of STIB 345 AB were grown in SIV rats. Rats were inoculated intraperitoneally with 10^6 trypanosomes, and killed on day 5.

Investigation of the influence of the temperature on the chromatin of procyclic culture forms

To investigate the effect of temperature, procyclic culture forms were cultivated as described above, but incubated for 1 week at 32 °C. After that period they were incubated at the following temperatures: 34, 35, 35·5, 36, 37 °C for various periods of time.

Purification of nuclei

Approximately 10 g liver from rats was homogenized in the presence of 1 mM phenylmethylsulphonylfluoride (PMSF) and the nuclei were purified by centrifugation through sucrose cushions (Thoma, Koller & Klug, 1979; Hecker & Gander, 1985). *T. b. brucei* STIB 345 AB were harvested, and $2–3 \times 10^{10}$ exponentially growing procyclic cells resuspended in hypotonic buffer containing 0·5 M 2-methyl-2,4-pentandiol (hexylene glycol), 2·5 mM $CaCl_2$, 1 mM PMSF. They were lysed by nitrogen cavitation at 25 bar (Shapiro & Doxsey, 1982), vortexed for 30 s and the isolated nuclei were washed in 90 mM suspension buffer, pH 7·4, containing 0·1 mM disodium ethylendiaminetetraacetic acid (Na_2EDTA) (Thoma *et al.* 1979; Hecker & Gander, 1985).

Rats were anaesthetized with methoxyfluorane. Bloodstream forms were collected by cardiac puncture and separated from the blood by a DEAE-52 cellulose column (Lanham & Godfrey, 1970). Trypanosomes were isolated by centrifugation at 1800 ***g*** for 20 min. Isolation of the nuclei was the same from this step on as for procyclic culture forms.

Preparation of soluble chromatin

Nuclei of *T. b. brucei* were digested with 0·2 units of micrococcal nuclease (Sigma, N-3755) per 20 A_{260} at 30 °C for 50 s. Nuclei of rat liver were digested with 0·4 units of micrococcal nuclease per 20 A_{260} at 37 °C for 50 s. The nuclei were centrifuged, and the chromatin solubilized by nuclear lysis in a hypotonic buffer containing 1 mM triethanolamine hydrochloride (TEACl) and 0·2 mM Na_2EDTA, pH 7·4. Insoluble material was removed by centrifugation (Thoma *et al.* 1979; Hecker & Gander, 1985). The preparation of soluble chromatin was identical for procyclic culture forms and for bloodstream forms.

Gradient analysis of the chromatin digest

Gradient analysis was done in 5·5–28·5% (w/v) 17 ml isokinetic sucrose gradients containing 5 mM TEACl, pH 7·4, 0·2 mM Na_2EDTA and 10 mM NaCl (Noll, 1969). Centrifugation was performed for 14 h at 25000 ***g*** in a Kontron TST 28/17 swing-out rotor. The gradients were monitored at 254 nm and the bottom fractions containing the larger fragments of soluble chromatin were used for analysis by electron microscopy (Thoma & Koller, 1981).

Electron microscopy

The fractions with the large chromatin fragments were divided into 4 aliquots and dialysed against 5 mM TEACl (pH 7·4), 0·2 mM Na_2EDTA containing 0, 10, 40 or 100 mM NaCl respectively for 4 h. Glutaraldehyde was then added to the dialysis buffer at a concentration of 0·2% (v/v) for trypanosome chromatin or 0·1% for rat liver chromatin, and samples were fixed for at least 15 h and prepared for EM observation (Thoma *et al.* 1979). The concentration of 0·2% glutaraldehyde for trypanosomes was chosen after an experimental investigation of the effect of different levels of glutaraldehyde.

Protein extraction from purified nuclei

Nuclei were resuspended in 0·25 M HCl (Elpidina, Zaitseva & Krasheninnikov, 1979) or in 5% (v/v) perchloric acid (PCA) or 350 mM NaCl in 10 mM TEACl, pH 7·4, (Sanders, 1977) and proteins extracted for 60 min under constant agitation. Insoluble material was pelleted at 4000 ***g*** for 5 min. The supernatant fractions, containing the histones or non-histones, were removed and dialysed against 1 mM TEACl, 0·2 mM Na_2EDTA, pH 7·4, and lyophilized.

All the preparations were carried out at 0–4 °C if not otherwise stated.

Triton acid urea polyacrylamide gel electrophoresis

Lyophilized proteins were dissolved in sample buffer containing 2·5 M urea, 3 % (v/v) 2-mercaptoethanol, 0·01 % (v/v) pyronine G, 0·9 M acetic acid and incubated at 100 °C for 5 min. The stacking gel contained 12 %, the resolution gel 15 % polyacrylamide and both 2·5 M urea, 0·9 M acetic acid and 0·38 % (v/v) Triton DF 16. After a first pre-run (25 mA, up to constant voltage), a second pre-run was performed with 80 μl of 1 M cysteamine in 0·9 M acetic acid per lane at 25 mA for 1 h, to scavenge free radicals and to prevent oxidation of methionine residues. Then the probes were loaded and separated (Alfageme *et al.* 1974).

SDS–tricine–PAGE

Samples of proteins from soluble chromatin were either lyophilized or precipitated in 25 % (v/v) trichloroacetic acid (TCA), pelleted at 15000 ***g***, washed with 100 % acetone and vacuum-dried. Samples were solubilized in sample buffer containing 4 % SDS, 12 % (w/v) glycerol, 50 mM Tris, 2 % (v/v) 2-mercaptoethanol, 0·01 % (w/v) Servablue G, pH 6·8, boiled and separated in a 17·6 % SDS–tricine–polyacrylamide resolution gel, with a 5 % stacking gel (Schägger & von Jagow, 1987).

Staining and destaining procedures

Coomassie brilliant blue. Gels were stained with 0·25 % (w/v) Coomassie brilliant blue R-250 in methanol:water:glacial acetic acid (5:5:1) for 1 h and destained with methanol:water:glacial acetic acid (4:5:1) for 1 h and (10:83:7) overnight, respectively.

Differential destaining with $FeCl_3$ of Coomassie-stained histones. Differential destaining with 0·1 M $FeCl_3$ in 20 % (v/v) ethanol was performed for 6 h (Spiker, Key & Wakim, 1976; Duschak & Cazzulo, 1990).

Bromophenol blue. Gels, fixed in methanol:water:acetic acid as described above were stained in 0·01 % (w/v) bromophenol blue in water (pH 3·0) for 24 h at room temperature, and differentially destained in 40 % (v/v) *n*-propanol for 24 h at 55 °C (Duschak & Cazzulo, 1990).

Micrococcal nuclease digestion and DNA agarose gel electrophoresis

Isolated nuclei of *T. b. brucei* procyclic culture forms as well as of bloodstream forms were digested with micrococcal nuclease, at concentrations of 0·1, 0·2 and 0·4 units per 20 A_{260} of nuclear suspension at 37 °C (Hewish & Burgoyne, 1973). Aliquots were withdrawn after 1, 4 and 10 min, and the digestion stopped with Na_2EDTA (final concentration 2·5 mM). DNA was prepared for gel electrophoresis, and samples of about 5 μg DNA were separated on a horizontal 1·5 % (w/v) agarose gel or on a linear 5·5 % polyacrylamide gel and stained with ethidium bromide according to methods described by Sambrook, Maniatis & Fritsch (1989).

RESULTS

Effect of gentamicin on procyclic culture forms

Procyclic culture forms are routinely cultivated in the presence of 10 μg gentamicin/ml of culture medium. In bacteria the antibiotic substance gentamicin interferes with mRNA translation and therefore leads to the synthesis of defective proteins. No influence of the antibiotic on the composition, structure and compaction of the chromatin could be seen, either in the absence of gentamicin or with a 5-fold higher concentration of gentamicin than is commonly used (50 μg/ml) (results not shown).

Structure and compaction pattern of soluble chromatin

Soluble chromatin of *T. b. brucei* bloodstream forms, centrifuged through a sucrose gradient containing 10 mM NaCl, dialysed against concentrations of 0, 10, 40, or 100 mM NaCl, and prepared for EM, showed condensation at increasing ionic strength (Fig. 1E–H). The compaction was more pronounced as compared to procyclic culture forms (Fig. 1I–M). However, solenoids (30 nm fibres) which are typical for rat liver chromatin (Fig. 1A–D), were not formed. Free linker DNA could barely be seen in rat liver chromatin (Fig. 1A–C), but was clearly visible in chromatin from both trypanosome stages at low salt concentrations (Fig. 1E–F and I–L). The nucleosomes of the chromatin filaments of procyclic culture forms were irregularly spaced (Fig. 1I). This was also seen in bloodstream forms but to a lesser extent (Fig. 1E), while chromatin of rat liver never showed such an irregular arrangement (Fig. 1A).

Triton acid urea PAGE

In triton acid urea gels, which separate proteins according to their hydrophobicity, core histones of trypanosomes migrated in four main complexes a, b, c, d and in a fast migrating, hydrophilic e-complex (Fig. 2, Lanes 2 and 3). Apart from the proteins of the d-regions, which had similar hydrophobic properties to H4 of higher eukaryotes (Fig. 2, Lane 1), all the histones of trypanosomes differed from those of higher eukaryotes. No proteins of higher eukaryotes can be seen in the e-region.

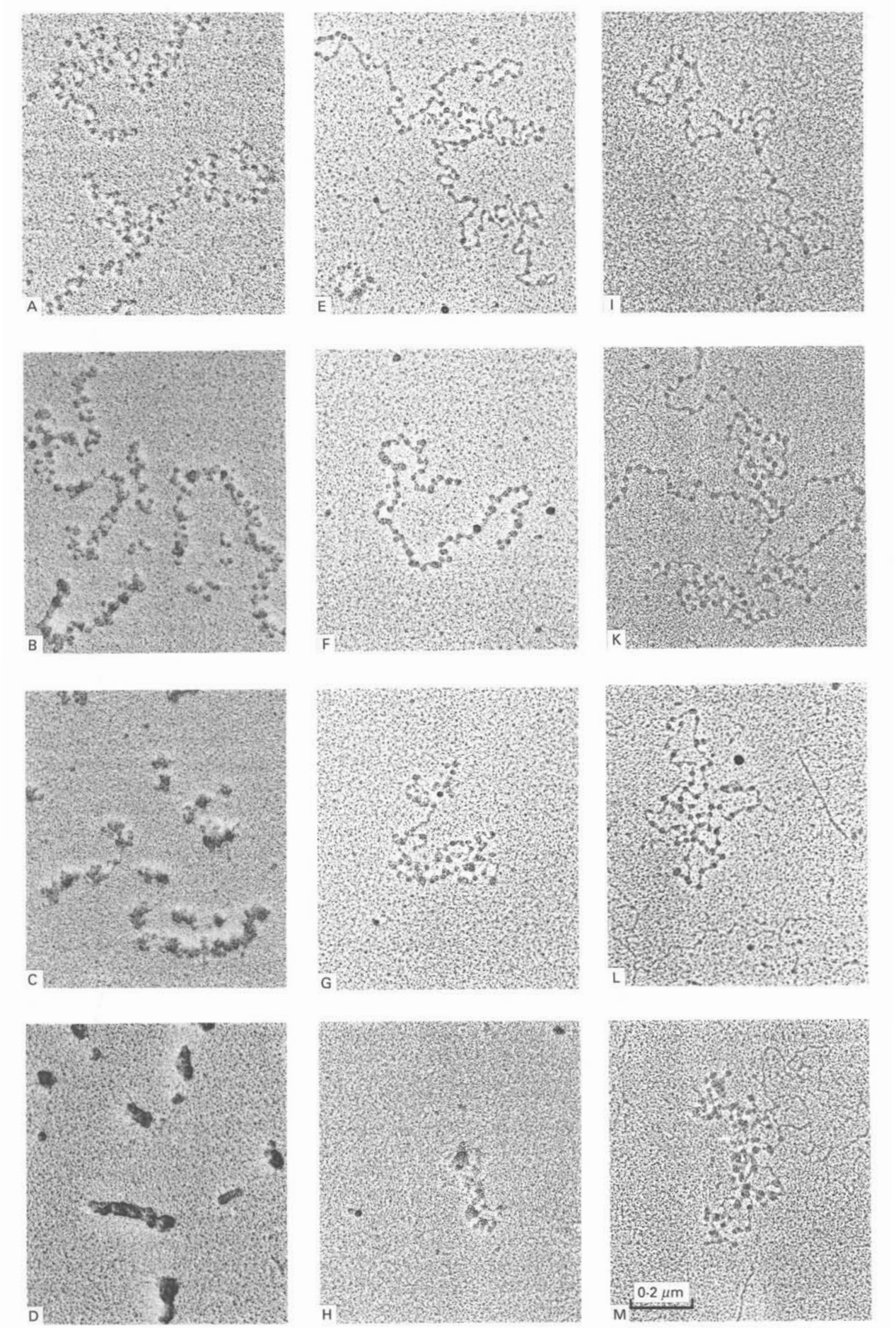

Fig. 1. For legend see opposite.

Differences in the number of bands, their position in the gel and their relative amount were seen between the histones of the two trypanosome stages. This was especially true for the e-region (Fig. 2, Lanes 2 and 3). All the proteins in the e-area could be selectively extracted with 5 % PCA (Fig. 2, Lanes 4 and 5). These proteins were metachromatically stained with Coomassie brilliant blue, which cannot be seen in black and white reproduction. Suspension of nuclei in 350 mM NaCl extracted non-histone proteins but not histone H1 of rats (Fig. 2, Lane 8). Proteins of the e-region were not extracted from nuclei with 350 mM NaCl, neither from procyclic culture forms (Fig. 2, Lane 6) nor from bloodstream forms (Fig. 2, Lane 7).

Selective destaining of histones

Histones of bloodstream forms, separated in Triton acid urea gels, were stained with 0·01 % bromophenol blue, and the destaining of the proteins in 40 % *n*-propanol was densitometrically analysed (Fig. 3A). Selective destaining of the proteins in the e-region could be seen after 6 h (Fig. 3B). The proteins in the b- and c-regions were destained after 24 h, the staining intensity of the d-region was reduced, and no destaining took place in the a-region (Fig. 3C). Procyclic culture forms showed the same destaining behaviour (not shown). Differential destaining of Coomassie brilliant blue-stained histones with 0·1 M $FeCl_3$ revealed a selective destaining of the e-region after 2 h (not shown).

SDS–tricine–PAGE

Histones of higher eukaryotes separated into a complex of 4 core histones and 2 H1 variants in SDS–tricine–PAGE (Fig. 4, Lane 1). Core-histones of trypanosomes and higher eukaryotes could be seen to differ in their electrophoretic mobility (Fig. 4, Lanes 1, 2 and 3). The strongest differences existed between rat liver histones H3 and H4 and their trypanosome counterparts a and d. Histones of bloodstream forms showed a different banding pattern compared to that of procyclic culture forms. In this high-resolution gel system, the histone b was separated into two variants in both trypanosome stages (b1 and b2) (Fig. 4, Lanes 2 and 3) and histone c shows three variants (c1, c2 and c3), but only in bloodstream forms (Fig. 4, Lane 3). Gels stained with Coomassie brilliant blue showed differences in the amounts of proteins b1 and b2 in procyclic culture forms and bloodstream forms (Fig. 4, Lanes 2 and 3). Neither procyclic culture forms nor bloodstream forms had any protein migrating in the H1 region of higher eukaryotes (Fig. 4, Lanes 1, 2 and 3). Proteins extracted with 5 % PCA migrated in the lower region of the core-histones and were metachromatically stained with Coomassie brilliant blue (Fig. 4, Lanes 4 and 5). These PCA-extractable proteins separated in four bands (1–4) in procyclic culture forms and in three bands (1′–3′) in bloodstream forms.

Among the HCl-extractable proteins of procyclic culture forms, only band 1 was clearly separated from the others, while bands 2–4 were partly overlaid by histone d. Only faint bands above and below that of histone d indicate their presence (Fig. 4, Lane 6). Positioning of the bands by densitometric tracing revealed that the PCA-extractable bands 1′, 2′ and 3′ of bloodstream forms were also visible in HCl extracts.

No significant differences could be seen between the two stages in proteins extractable in 0·25 M HCl or DNA-associated proteins derived from soluble chromatin (Fig. 4, Lanes 2, 3, 6 and 7).

Nucleosomal pattern after micrococcal nuclease digestion

Ladders of DNA fragments ranging from oligonucleosomes to mononucleosomes were visible in agarose gels when chromatin of *T. b. brucei* procyclic culture forms were digested with micrococcal nuclease for 1 min (Fig. 5A, Lane 3). Chromatin of bloodstream forms was digested to a lesser extent, and that of rat liver still less (Fig. 5A, Lanes 4 and 1). After 10 min digestion, nucleosome ladders were still found for rat liver (Fig. 5A, Lane 9), whereas chromatin of both trypanosome stages was more extensively digested, mainly to mononucleosomes and dinucleosomes (Fig. 5A, Lanes 7 and 8). The strongest differences in the digestion patterns between procyclic culture forms and bloodstream forms were seen after 4 min (Fig. 5A, Lanes 5 and 6). Mixed preparations of DNA fragments of procyclic culture forms and bloodstream forms after 1 min digestion (Fig. 5A, Lane 2) showed nucleosomal ladders identical to those of single preparations (Fig. 5A, Lanes 3 and 4), which demonstrates that the linker length is identical for both life-cycle stages of *T. b. brucei*. The differences were not affected by changes in the enzyme concentration (0·1, 0·2 and 0·4 units micrococcal nuclease per 20 A_{260} nuclei).

The same samples separated on a high-resolution polyacrylamide gel showed mainly mononucleo-

Fig. 1. Soluble chromatin of *Trypanosoma brucei brucei* and of rat liver from a sucrose gradient containing 10 mM NaCl, dialysed against concentrations of 0 mM NaCl (A, E, I), 10 mM NaCl (B, F, K), 40 mM NaCl (C, G, L) and 100 mM NaCl (D, H, M). Rat liver (A–D); bloodstream forms (E–H); procyclic culture forms (I–M).

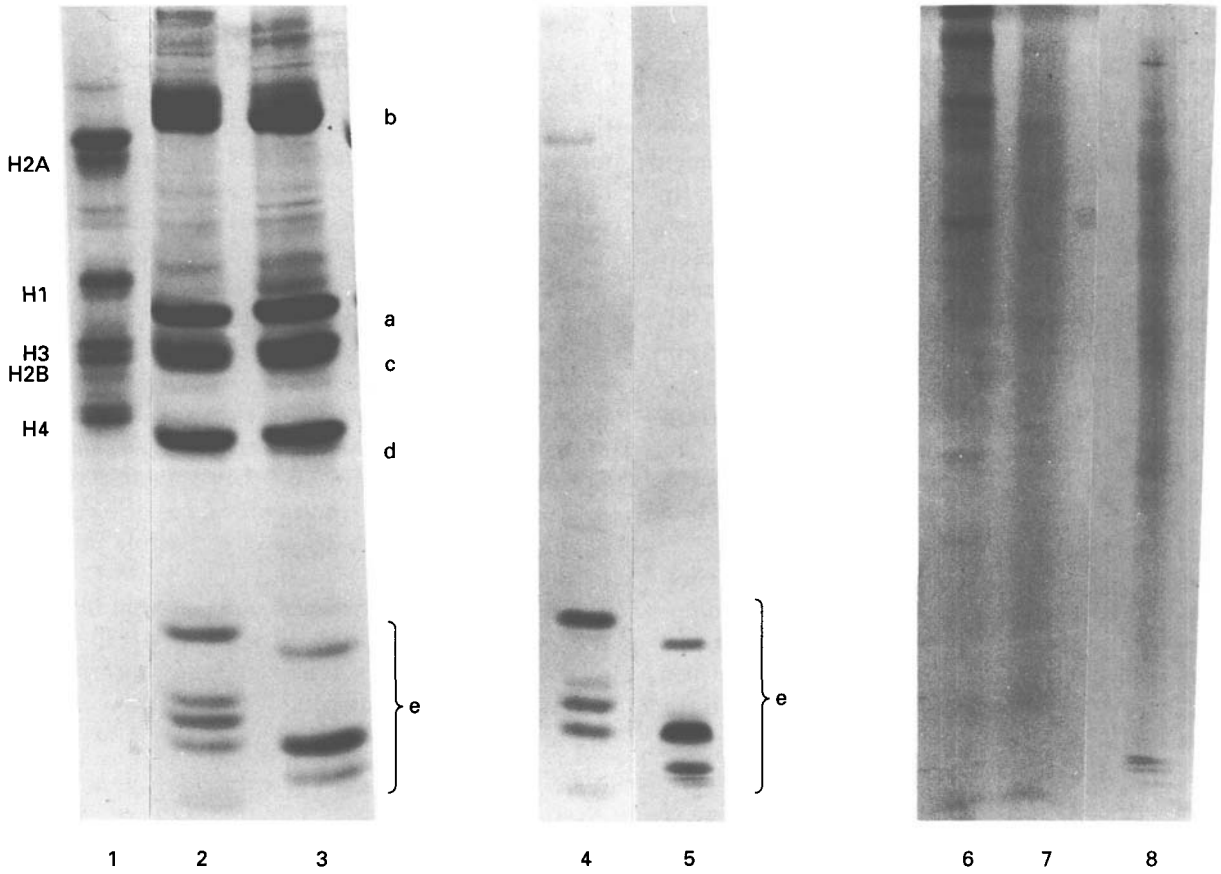

Fig. 2. Histone analysis in Triton acid urea PAGE. Lane 1: calf thymus histones. HCl-extracted histones of nuclei of *Trypanosoma brucei brucei* procyclic culture forms (Lane 2) and of bloodstream forms (Lane 3); 5% perchloric acid-extracted histones of procyclic culture forms (Lane 4) and bloodstream forms (Lane 5). Non-histone proteins extracted with 350 mM NaCl from procyclic culture forms (Lane 6), bloodstream forms (Lane 7) and rat liver (Lane 8).

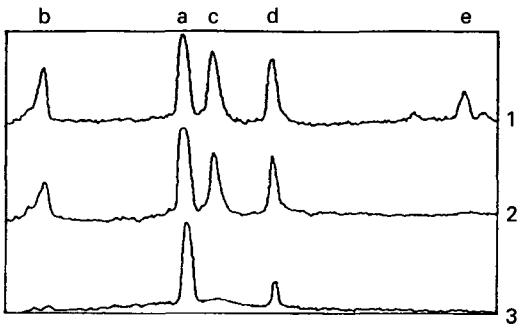

Fig. 3. Selective destaining of bromophenol blue stained histones. Histones of *Trypanosoma brucei brucei* bloodstream forms, separated on a Triton DF-16 acid urea gel and stained with bromophenol blue, were treated with 40% *n*-propanol, which destains selectively histone H1, H2A and H2B. Densitometric Trace 1: before destaining; Trace 2: after 6 h; Trace 3 after 24 h of destaining. After 6 h the e-bands and after 24 h the b and c-bands, which are the counterparts of H2A and H2B in higher eukaryotes, are destained.

some-sized and traces of dinucleosome-sized DNA fragments in procyclic culture forms after digestion for 10 min (Fig. 5B, Lane 2). Bloodstream forms showed fragments up to four nucleosomes at the same digestion time (Fig. 5B, Lane 3). The mean size of the mononucleosome fragments was larger for the bloodstream forms than for procyclic culture forms after 10 min digestion. The centre of the corresponding band in bloodstream forms was found above the 154 bp fragment of the DNA marker, while the centre of the band of the procyclic culture forms was situated in the region of this marker fragment. After 30 min digestion, DNA fragments were found in the region of 154 bp as well as below this marker for both trypanosome stages (Fig. 5B, Lanes 4 and 5).

DISCUSSION

The identification of histone H1 can be difficult, since it is the most variable of all histones. In several cases, the original reports stated that H1 was absent (van Holde, 1989) and only careful choice of experimental conditions, use of protease inhibitors and specially adapted gel systems allowed the demonstration of its presence. In *T. b. brucei*, a histone H1-like protein was postulated to be absent (Hecker & Gander, 1985; Hecker *et al.* 1989; Bender

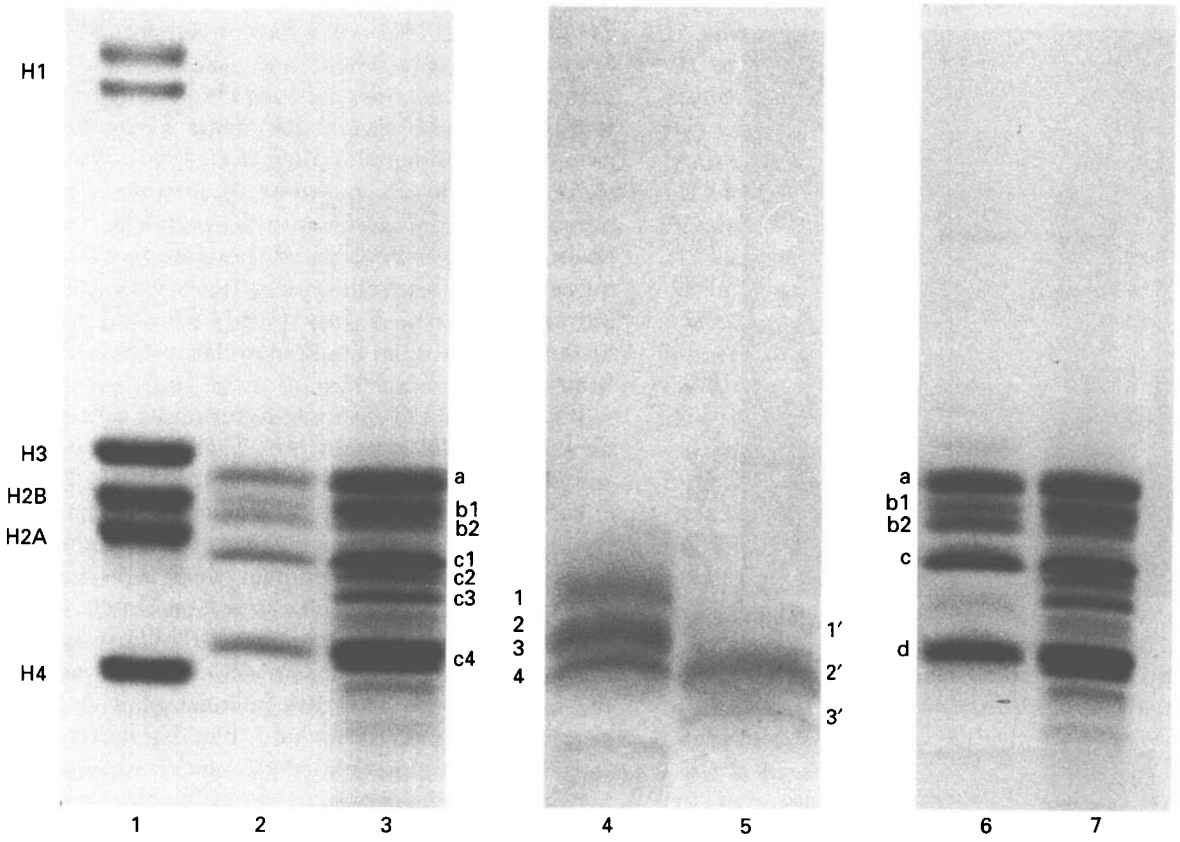

Fig. 4. Histone analysis in SDS–tricine–PAGE. Lane 1: calf thymus histones. HCl-extracted histones of *Trypanosoma brucei brucei* procyclic culture forms (Lane 2) and bloodstream forms (Lane 3); 5% perchloric acid-extracted histones of bloodstream forms (Lane 4) and of procyclic culture forms (Lane 5); histones from soluble chromatin of procyclic culture forms (Lane 6) and bloodstream forms (Lane 7).

et al. 1992*a–d*), although it was shown in other trypanosomatids (Toro & Galanti, 1988, 1990; Duschak & Cazzulo, 1990).

In the present paper, proteins called e-region were seen in *T. b. brucei* for the first time. We postulate that the proteins of the e-region are histone H1-like proteins. This is supported by their extractability with 5% PCA, a typical feature of histone H1 of higher eukaryotes (Sanders, 1977). If these proteins are stained with Coomassie Brilliant blue, they show metachromasia; a reddish colour, which is a sign of a high lysine content (Duhamel, Meezan & Brendel, 1980). Additional evidence comes from destaining experiments with *n*-propanol (Duschak & Cazzulo, 1990) and $FeCl_3$ (Spiker *et al.* 1976; Duschak & Cazzulo, 1990), which destains histone H1. Using both methods the e-region is destained first. Additionally, due to their non-extractability with 350 mM NaCl, it could be ruled out that these proteins were non-histones (Johns, 1982). Antibodies directed against H1 of sea urchin showed cross-reactivity to the fast migrating proteins of *T. cruzi* (Toro & Galanti, 1988), the counterparts of the e-bands in *T. b. brucei*. It has been shown by means of amino acid analysis that these proteins of *T. cruzi* are histone H1-like proteins (G. C. Toro & N. Galanti, personal communication).

Triton binds to histones proportionally to their degree of hydrophobicity and thereby reduces the electrophoretic mobility (Hardison & Chalkley, 1978). The histone H1-like proteins of *T. b. brucei* are strongly hydrophilic and very fast migrating in Triton acid urea gels. The banding pattern in Triton DF-16 gels of histones of *T. cruzi*, *T. b. brucei* and *Crithidia fasciculata* is similar and all species possess fast migrating hydrophilic histone H1-like proteins. However, the banding pattern of the fast migrating bands in trypanosomatids differs. Differences in the number, amount and position of the H1-like proteins can also be seen, when the two stages of the life-cycle of *T. b. brucei* are compared.

Histone H1-like proteins of *T. b. brucei* run out of Triton and acid urea gels owing to their high mobility unless specially adapted systems are used. In SDS gels they can only be detected if the linear SDS–tricine–PAGE according to the method of Schägger & von Jagow (1987), optimized for highest resolution in the region of the core histones, is used. The resolution in this gel system is superior to that in gradient gels according to Laemmli (1970) which were originally used by Hecker & Gander (1985), and Bender *et al.* (1992*a*). The strong hydrophilic character and the small molecular weight of the H1-like proteins of *T. b. brucei* are the reasons why

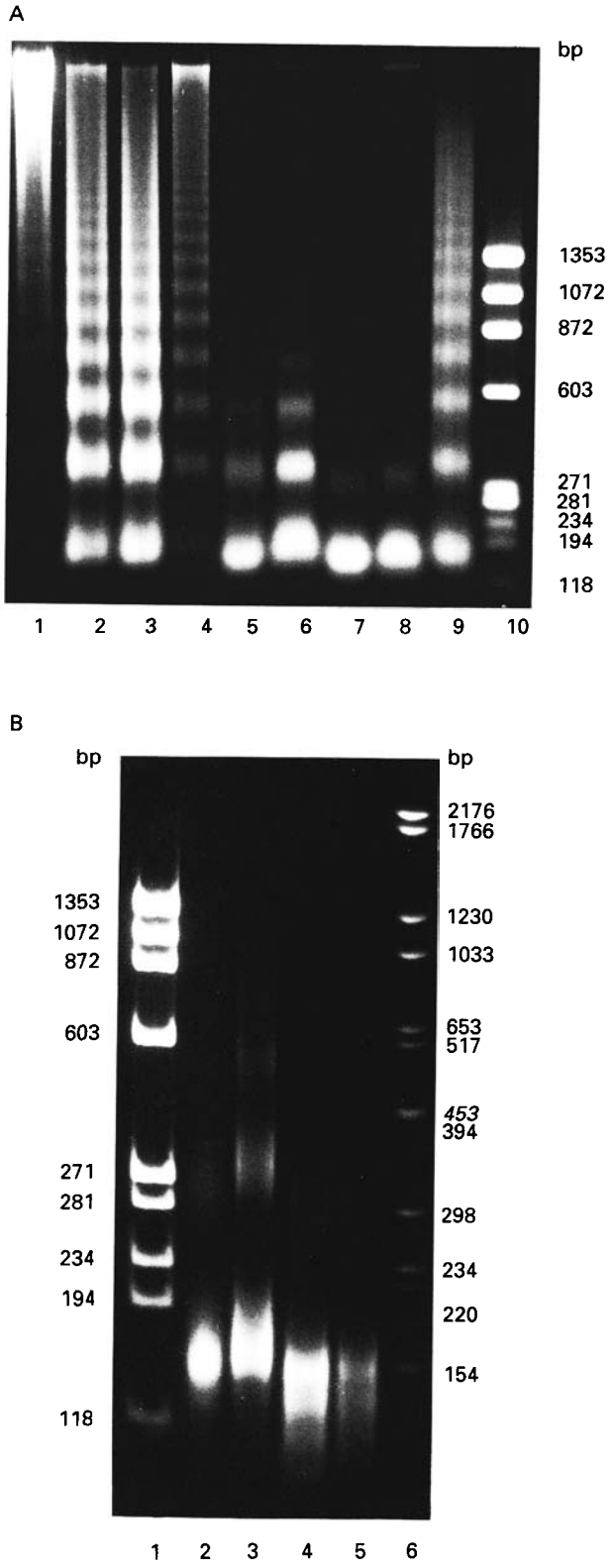

Fig. 5. (A) Separation of DNA fragments in a 1·5% agarose gel. Micrococcal nuclease digestion patterns of chromatin of rat liver, *Trypanosoma brucei brucei* procylic culture and bloodstream forms. Marker: HAE III fragments of Φ X 174 RF DNA (Lane 10). Lanes 1 and 9: rat liver; Lanes 3, 5 and 7: procyclic culture forms; Lanes 4, 6 and 8: bloodstream forms; Lane 2: mixture of procyclic culture and bloodstream forms. Digestion times: Lanes 1–4: 1 min; Lanes 5 and 6: 4 min; Lanes 7–9: 10 min. (B) Separation of DNA fragments in a 5·5% polyacrylamide gel. Markers: HAE III fragments of Φ X 174 RF DNA (Lane 1) and DNA fragments of pBR328 cleaved with *Bgl* I and *Hinf* I (Lane 6). Procyclic culture forms (Lanes 2 and 4) and bloodstream forms (Lanes 3 and 5) after 10 or 30 min digestion. The centre of the band of mononucleosome fragments of bloodstream forms (Lane 3) is above the 154 bp marker fragments (Lane 6), while one of the procyclic culture forms (Lane 2) is in the region of the latter.

Hecker & Gander (1985), Hecker *et al.* (1989) and Bender *et al.* (1992*a–d*), failed to demonstrate their existence.

In higher eukaryotes, histone H1 is composed of a central hydrophobic globular domain and two adjacent hydrophilic tails (Allan *et al.* 1980). We could show that the PCA extracted histone H1-like proteins of *T. b. brucei* run in the region of the core histones in SDS–PAGE and therefore they may be quite small. This finding is in line with a study of Hayashi, Hayashi & Iwai (1987) who described a histone H1 with a small globular part in *Tetrahymena*.

If the N- and C-terminal hydrophilic tails of the histone H1-like proteins in *T. b. brucei* are of a similar length, compared to those of higher eukaryotes, then the hydrophobic globular region would occupy only a small proportion. The globular part of histone H1 is the domain which is responsible for the binding to the nucleosome (van Holde, 1989). A small sized globular domain could indicate a weak interaction of the trypanosome H1-like proteins with the nucleosomes. The core histones of *T. b. brucei* procyclic culture forms and bloodstream forms, separated according to the molecular weight or to the hydrophobicity, are similar, but differ in the position and number of the bands as compared to higher eukaryotes. This result is in line with differences reported for the amino acid composition between *T. b. brucei* procyclic culture forms and higher eukaryotes (Bender *et al.* 1992*a*). By selective destaining with *n*-propanol, we were able to show that the proteins of the bloodstream forms, which migrate in the positions of histones b and c in Triton gels are the counterparts of H2A and H2B (Duschak & Cazzulo, 1990). Life-cycle specific variants and/or modifications can be seen in histone b, which is resolved in two proteins b1 and b2 in SDS–tricine–PAGE, as well as in histone c, which is resolved only in bloodstream forms into three bands c1, c2 and c3. Differences in the amount between the two bands of protein b occur between the two trypanosome stages and can be seen in SDS–tricine as well as in Triton acid urea gels.

Weak interaction and instability within the chromatin enhances the accessibility of the linker DNA towards micrococcal nuclease and this leads to a

faster digestion of the chromatin of the procyclic culture forms (Hecker *et al.* 1989) relative to higher eukaryotes. Bloodstream forms appear to be intermediate. Digestion is slower than in procyclic culture forms, but still faster than for higher eukaryotes. There is a clear digestion barrier in higher eukaryotes in the presence of H1 (Telford & Stewart, 1989), which cannot be seen in procyclic culture forms (Hecker *et al.* 1989). After digestion for 10 min, bloodstream forms yield larger mononucleosome-sized DNA fragments than the procyclic culture forms. These larger fragments are no longer seen after 30 min digestion. A weak digestion barrier in bloodstream forms may be due to a somewhat stronger interaction between histone H1-like proteins and the DNA, as compared to the procyclic culture form.

Genetically inactive chromatin of higher eukaryotes forms 30 nm fibres (solenoids) at 100 mM NaCl. The formation of the 30 nm fibres depends on the presence of the lysine-rich histone H1. In the absence of histone H1, nucleosome filaments do not condense to solenoids, and form only loose aggregates (Thoma *et al.* 1979). The spacing of the nucleosomes on the DNA is irregular and nucleosome sliding occurs (Stein & Bina, 1984).

Chromatin of *T. b. brucei* procyclic culture forms, prepared under identical conditions to rat liver chromatin, is organized in the form of nucleosome filaments. Previous studies on the ultrastructure of the chromatin of *T. b. brucei* procyclic culture forms revealed a bad conservation of the nucleosome filaments (Hecker & Gander, 1985). The nucleosomes were irregularly spaced and at increasing ionic strength only a slight aggregation of the nucleosomes could be seen (Hecker & Gander, 1985). The interactions of histones and DNA are weak and are easily destabilized by experimental manipulation (Hecker & Gander, 1985; Hecker *et al.* 1989; Bender *et al.* 1992*c*).

To try to improve the preservation of the chromatin filaments of trypanosomes, the effect of increasing the concentration of glutaraldehyde for the fixation of soluble chromatin for EM studies was investigated. The results obtained with 0·2% glutaraldehyde were superior to those with the 'conventional' concentration of 0·1% (Thoma *et al.* 1979; Hecker & Gander, 1985). Chromatin prepared with 0·5% glutaraldehyde showed an identical condensation behaviour to the one fixed with 0·2%.

The stability of chromatin depends on the nature and strength of the DNA–protein interactions (Yager, McMurray & van Holde, 1989). In contrast to the chromatin of procyclic culture forms, the chromatin of bloodstream forms shows a stronger condensation at increasing ionic strength but it does not form 30 nm fibres. The compaction pattern of soluble chromatin of *T. b. brucei* bloodstream forms is structurally similar to that of *T. cruzi* epimastigote culture forms (Hecker & Gander, 1985). Histone H1-like proteins were shown for *T. cruzi* by Toro & Galanti (1988, 1990). Hecker & Gander (1985) could induce a higher condensation level in *T. cruzi* with the addition of a histone H1-containing fraction from rat liver chromatin. This result suggested an H1 binding site in *T. cruzi* chromatin. Additionally, Bender (1991) showed that a binding site for histone H1 exists in the chromatin of *T. b. brucei* procyclic culture forms.

The finding of Hecker & Gander (1985), that no higher condensation level was achieved in procyclic culture forms of *T. b. brucei* by the addition of H1 of rats, is most probably due to the damage of the chromatin having been introduced by the experimental procedure to make soluble chromatin and the concomitant dissociation of nucleosomes from the DNA. A subsequent reconstitution is no longer possible. The result is the formation of 'clumps on a string' with large stretches of free DNA.

The histone patterns of the e-region of procyclic culture forms and bloodstream forms are different in number and position of bands and in their relative amounts. Modifications of the histones may influence histone–histone interactions as shown by Simpson (1981) for higher eukaryotes, and may contribute to a higher stability of the core particle especially in bloodstream forms. Effects of histone modifications were shown on the condensation behaviour of nucleosome filaments into higher-order structures for higher eukaryotes (Marion *et al.* 1985).

The different histone patterns of the two compared trypanosome stages are most likely the reason for the different condensation behaviour of their chromatin. The H1-like proteins of procyclic culture forms seem to interact very weakly with the DNA and this could be the reason why no condensation takes place, since most of these proteins dissociate during the preparation of soluble chromatin. This would also explain the poor preservation of the nucleosome filaments of procyclic culture forms at 0 mM NaCl, which is in accordance with the results of Hecker *et al.* (1989), who showed that chromatin of procyclic culture forms is very unstable. As chromatin from both stages of the life-cycle was prepared under identical conditions, observed differences of the chromatin parameters must be intrinsic differences between the life-stages of *T. b. brucei*.

We wondered why trypanosomes undergo changes in their histones during their life-cycle and suggest that the stronger DNA–histone interaction in bloodstream forms might be a requirement for a stabilization of the chromatin at the higher temperatures of the mammalian host. When procyclic culture forms were cultivated above 35 °C, they stopped cell division and died after approximately 3 days. All chromatin parameters investigated, including the histone pattern in gels, were those usually found in procyclic culture forms. The fact that procyclic

culture forms cannot produce the histone H1 pattern and core histone variants of modifications of bloodstream forms and die after several days when cultivated between 35 and 37 °C, supports this hypothesis. Since trypanosomes derive from free-living flagellates (Lumsden & Evans, 1976), the ability of bloodstream forms to support higher temperatures might be regarded as an adaptation to parasitism.

It can be concluded that *T. b. brucei* contains a full set of histones, including H1-like proteins, and that the poor condensation of its chromatin is not due to the absence of H1, but most probably to peculiar properties of H1 and core histones. Despite common features, the histones and therefore various properties of the nuclear chromatin are quite different between bloodstream and procyclic culture forms. As a consequence, the latter can no longer be regarded as the only model for all stages of the life-cycle of *T. b. brucei*.

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REFERENCES

ALFAGEME, C. R., ZWEIDLER, A., MAHOWALD, A. & COHEN, L. H. (1974). Histones of Drosophila embryos. *Journal of Biological Chemistry* **249**, 3729–36.

ALLAN, J., HARTMAN, P. G., CRANE-ROBINSON, C. & AVILES, F. X. (1980). The structure of histone H1 and its location in chromatin. *Nature, London* **288**, 675–9.

BENDER, K. (1991). Biochemical and structural aspects of the nuclear chromatin of procyclic *Trypanosoma brucei brucei*. Ph.D. thesis, University of Basel.

BENDER, K., BETSCHART, B., SCHALLER, J., KÄMPFER, U. & HECKER, H. (1992*a*). Biochemical properties of histone-like proteins of procyclic *Trypanosoma brucei brucei*. *Acta Tropica* **50**, 169–84.

BENDER, K., BETSCHART, B., SCHALLER, J., KÄMPFER, U. & HECKER, H. (1992*b*). Sequence differences between histones of procyclic *Trypanosoma brucei brucei* and higher eukaryotes. *Parasitology* **105**, 97–104.

BENDER, K., BETSCHART, B. & HECKER, H. (1992*c*). Histone–DNA interactions in the chromatin of procyclic *Trypanosoma brucei brucei*. *Parasitology Research* **87**, 495–500.

BENDER, K., BETSCHART, B., MARION, C., MICHALON, P. & HECKER, H. (1992*d*). Structural differences between the chromatin of procyclic *Trypanosoma brucei brucei* and higher eukaryotes as probed by immobilized trypsin. *Acta Tropica* **52**, 69–78.

BRUN, R. & SCHÖNENBERGER, M. (1979). Cultivation and *in vitro* cloning of *Trypanosoma brucei* in a semi-defined medium. *Acta Tropica* **36**, 289–92.

DUHAMEL, R. C., MEEZAN, E. & BRENDEL, K. (1980). Metachromatic staining with Coomassie-Brilliant-Blue R-250 of the proline-rich calf thymus histone H1. *Biochimica et Biophysica Acta* **626**, 432–42.

DUSCHAK, V. G. & CAZZULO, J. J. (1990). The histones of the insect trypanosomatid, *Crithidia fasciculata*. *Biochimica et Biophysica Acta* **1040**, 159–66.

ELPIDINA, E. N., ZAITSEVA, G. N. & KRASHENINNIKOV, J. A. (1979). Histones from *Trypanosoma lewisi* nuclei. *Biokhimiya* **44**, 1830–41.

HAMM, B., SCHINDLER, A., MECKE, D. & DUSZENKO, D. (1990). Differentiation of *Trypanosoma brucei* bloodstream trypomastigotes from long slender to short stumpy-like forms in axenic culture. *Molecular and Biochemical Parasitology* **40**, 13–22.

HARDISON, R. & CHALKLEY, R. (1978). Polyacrylamide gel electrophoretic fractionation of histones. *Methods in Cell Biology* **17**, 235–51.

HAYASHI, T., HAYASHI, H. & IWAI, K. (1987). *Tetrahymena* histone H1. Isolation and amino acid sequence lacking the central hydrophobic domain conserved in other H1 histones. *Journal of Biochemistry* **102**, 369–76.

HECKER, H. & GANDER, E. S. (1985). The compaction pattern of the chromatin of trypanosomes. *Biology of the Cell* **53**, 199–208.

HECKER, H., BENDER, K., BETSCHART, B. & MODESPACHER, U. P. (1989). Instability of the nuclear chromatin of procyclic *Trypanosoma brucei brucei*. *Molecular and Biochemical Parasitology* **37**, 225–34.

HEWISH, D. R. & BURGOYNE, L. A. (1973). Chromatin sub-structure. The digest of chromatin DNA at regularly spaced sites by a nuclear deoxyribonuclease. *Biochemical and Biophysical Research Communication* **52**, 504–10.

JOHMANN, C. A. & GOROVSKY, M. A. (1976). Purification and characterisation of histones associated with the macronucleus of *Tetrahymena*. *Biochemistry* **15**, 1249–56.

JOHNS, E. W. (1982). Chapter 1. In *The HMG Chromosomal Proteins* (ed. Johns, E. W.), pp. 1–7. New York: Academic Press.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–5.

LANHAM, S. M. & GODFREY, D. G. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-Cellulose. *Experimental Parasitology* **28**, 521–34.

LUMSDEN, W. H. R. & EVANS, D. A. (1976). *Biology of the Kinetoplastida*, Vol. I. New York: Academic Press.

MARION, C., MARTINAGE, A., TIRARD, A., ROUX, B., DAUNE, M. & MAZEN, A. (1985). Histone phosphorylation in native chromatin induces local structural changes as probed by electric birefringence. *Journal of Molecular Biology* **186**, 367–79.

NOLL, H. (1969). An automatic high-resolution gradient analyzing system. *Analytical Biochemistry* **27**, 130–49.

SAMBROOK, J., MANIATIS, P. T. & FRITSCH, E. F. (1989). *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

SANDERS, C. (1977). A method for the fractionation of the high-mobility group non-histone proteins. *Biochemical and Biophysical Research Communications* **78**, 1034–42.

SCHÄGGER, H. & VON JAGOW, G. (1987). Tricine–sodium–dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry* **166**, 368–79.

SHAPIRO, S. Z. & DOXSEY, S. J. (1982). Purification of nuclei from a flagellate protozoan, *Trypanosoma brucei*. *Analytical Biochemistry* **127**, 112–15.

SIMPSON, R. T. (1981). Modulation of nucleosome structure by histone subtypes in sea urchin embryos. *Proceedings of the National Academy of Sciences, USA* **78**, 6803–7.

SPIKER, S., KEY, J. L. & WAKIM, B. (1976). Identification and fractionation of plant histones. *Archives of Biochemistry and Biophysics* **176**, 510–18.

STEIN, A. & BINA, M. (1984). A model chromatin assembly system: factors affecting nucleosome spacing. *Journal of Molecular Biology* **178**, 341–63.

TELFORD, D. J. & STEWART, B. W. (1989). Micrococcal nuclease: its specificity and use for chromatin analysis. *International Journal of Biochemistry* **21**, 127–37.

THOMA, F., KOLLER, TH. & KLUG, A. (1979). Involvement of histone H1 in the organization of the nucleosome and the salt-dependent superstructures of chromatin. *Journal of Cell Biology* **83**, 403–24.

THOMA, F. & KOLLER, TH. (1981). Unravelled nucleosomes, nucleosome beads and higher-order structures of chromatin: influence of non-histone components and histone H1. *Journal of Molecular Biology* **149**, 709–33.

TORO, G. C. & GALANTI, N. (1988). H1 histone and histone variants in *Trypanosoma cruzi*. *Experimental Cell Research* **174**, 16–24.

TORO, G. C. & GALANTI, N. (1990). *Trypanosoma cruzi* histones. Further characterization and comparison with higher eukaryotes. *Biochemistry International* **21**, 481–90.

TORO, G. C., WERNSTEDT, C., MEDINA, C., JARAMILLO, N., HELLMANN, U. & GALANTI, N. (1992). Extremely divergent histone-H4 sequence from *Trypanosoma cruzi*–evolutionary implications. *Journal of Cellular Biochemistry* **49**, 266–71.

VAN HOLDE, K. E. (1989). Chromatin. In *Springer Series in Molecular Biology* (ed. Rich, A.), pp. 168–80 and pp. 317–43. New York: Springer Verlag.

VICKERMAN, K. & PRESTON, T. M. (1970). Spindle microtubules in the dividing nuclei of trypanosomes. *Journal of Cell Science* **6**, 365–83.

YAGER, T. D., MCMURRAY, T. & VAN HOLDE, K. E. (1989). Salt-induced release of DNA from nucleosome core particles. *Biochemistry* **28**, 2271–81.