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Biochemical and functional characterization of histone H1-like proteins in procyclic *Trypanosoma brucei brucei*

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Abstract. Four variants and/or posttranslational modifications of histone H1-like proteins of *Trypanosoma brucei brucei* procyclic culture forms were extracted with 0.25 *N* HCl from isolated nuclei and analyzed by two-dimensional gel electrophoresis. The amino acid composition of these proteins, their ability to space nucleosomes regularly and to induce salt-dependent condensation of the chromatin indicated their histone H1 nature. On the other hand, the histone H1-like proteins clearly differed from their higher-eukaryote counterparts by their weak interaction with DNA under low-salt conditions. As a consequence, intact nucleosome filaments were prepared according to a new preparation protocol especially adapted to the unstable chromatin of *T. b. brucei*. Our results indicate that the biochemical properties of the histone H1-like proteins contribute to the structural and functional differences between the chromatin of procyclic *T. b. brucei* and that of higher eukaryotes.

Histone H1 plays a role in the formation of chromatin structure at the level of the nucleosome filament and of the higher-order structures of the chromatin fiber in higher eukaryotes (Thoma et al. 1979; Crane-Robinson 1985; Widom 1989). In contrast to histones H3 and H4, histone H1 is little conserved throughout the animal and plant kingdoms. The biochemical heterogeneities reported for H1 in higher- and lower-eukaryote organisms are attributable to genetic variation and/or to postsynthetic covalent modifications (Gorovsky et al. 1974; Caplan 1975; Goff 1976; Pastink et al. 1979; van Holde 1989; Duschak and Cazzulo 1990; Toro et al. 1993).

It was considered by some workers that histone H1 was not present at all in trypanosomes (Elpidina et al. 1979; Rubio et al. 1980; Hecker and Gander 1985; Hecker et al. 1989; Bender et al. 1992a–d). On the other hand, Toro and Galanti (1988, 1990), Duschak and Cazzulo (1990), and Toro et al. (1993) postulated the presence of histone H1-like proteins in *Trypanosoma cruzi* and in *Crithidia fasciculata*. Recently, on the basis of electron microscopy, extractability, and gel-electrophoretic properties, Schlimme et al. (1993) described proteins with properties characteristic of histone H1 in *Trypanosoma brucei brucei*. The histone H1-like proteins of two stages of the life cycle, namely, bloodstream forms and procyclic culture forms, differed in the number of bands and their position in the gels as well as in their relative amounts. These proteins show a migration pattern in Triton acid-urea gels similar to that of the histone H1-like proteins of *T. cruzi* (Toro and Galanti 1988, 1990). On the other hand, their gel-electrophoretic properties are distinct from those of the histone H1 of higher eukaryotes.

Significant differences are present in the chromatin of procyclic *T. b. brucei* and of rat liver under the experimental conditions normally used for the isolation of chromatin from higher eukaryotes. The DNA-protein interactions are less stable in the trypanosome as compared with higher eukaryotes (Hecker et al. 1989). No salt-dependent condensation into higher-order structures could be seen in the chromatin of procyclic *T. b. brucei*, whereas rat-liver chromatin condensed into a 30 nm fiber (Hecker and Gander 1985; Schlimme et al. 1993). The observed structural and functional differences in the chromatin were explained in terms of special properties of the core histones (Hecker and Gander 1985; Hecker et al. 1989; Bender et al. 1992a–d). On the other hand, Schlimme et al. (1993) attributed the failure of the nucleosome filaments of procyclic *T. b. brucei* to condense to the weak interaction of the histone H1-like proteins with the DNA. It was supposed that these proteins are easily lost during the preparation of the soluble chromatin.

In the present paper, the histone H1-like proteins of procyclic *T. b. brucei* were characterized by their migration pattern in two-dimensional gel electrophoresis and by their amino acid composition. Their interaction with the DNA was investigated to assess their influence on

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the structure and function of the chromatin of procyclic *T. b. brucei.*

Materials and methods

Trypanosoma brucei brucei STIB 345 AB procyclic culture forms were cultivated in SDM 79 medium containing 10% heat-inactivated fetal bovine serum, 10 µg gentamicin/ml, and 10 µg hemin/ml (Brun and Schönenberger 1979).

Purification of nuclei

Nuclei were isolated from $2.5–4.5 \times 10^{10}$ exponentially growing procyclic cells by a method involving lysis by nitrogen cavitation at 23 bar as described by Shapiro and Doxsey (1982) and Schlimme et al. (1993).

Protein extraction from purified nuclei

Nuclei were resuspended in 0.25 *N* HCl (Elpidina et al. 1979) or in 5% (v/v) perchloric acid (PCA; Sanders 1977) and proteins were extracted for 60 min under constant agitation as described by Schlimme et al. (1993).

Triton acid-urea 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, and 0.9 *M* acetic acid and were prepared for gel electrophoresis according to Shmatchenko and Varshavsky (1978). The gels contained 2.5 *M* urea, 0.9 *M* acetic acid, and 0.38% (v/v) Triton DF 16 as well as 12% polyacrylamide in the stacking gel and 15% polyacrylamide in the separating gel. After a first prerun (25 mA, up to constant voltage), a second prerun was performed with 70 µl 1 *M* cysteamine in 0.9 *N* acetic acid per lane at 25 mA for 1 h. The samples were separated according to the method of Alfageme et al. (1974).

Acid-urea gel electrophoresis

Lyophilized proteins were dissolved and prepared for gel electrophoresis as described above. Gels containing 0.9 *N* acetic acid and 2.5 *M* urea in 12% polyacrylamide (stacking gel) or 15% polyacrylamide (separating gel) were prerun in 0.9 *N* acetic acid at 20 mA for 2 h. The samples were separated at 25 mA for 5 h (Panyim and Chalkley 1969; Shmatchenko and Varshavsky 1978).

Two-dimensional gel electrophoresis

In the first dimension, histones were separated in Triton acid-urea gels or in acid-urea gels as described above. A strip of gel from the first dimension was equilibrated in buffer containing 0.1 *M* TRIS (pH 8.3), 0.1 *M* *N*-[TRIS-(hydroxymethyl)-methyl]-glycine (Tricine), and 0.1% sodium dodecyl sulfate (SDS). The histones were separated in the second dimension in a 17.6% SDS-Tricine-polyacrylamide separating gel with a 5% stacking gel (Schägger and von Jagow 1987).

Gels were stained with 0.25% (w/v) Coomassie brilliant blue R-250 in methanol:water:glacial acetic acid (5:5:1, by vol.) containing 10% glutaraldehyde for 1 h and were destained with methanol:water:glacial acetic acid (4:5:1, by vol.) for 1 h and overnight (10:83:7, by vol.), respectively.

Isolation of histones for amino acid analysis

Histones separated in Triton acid-urea gels as described above were used for the electrophoretic blotting. After electrophoresis, the gels were soaked in 0.7% acetic acid (transfer solution) for 5 min. A polyvinylidenedifluoride (PVDF) membrane was rinsed with 100% methanol for 5 s, immersed in water for 2 min, and then equilibrated in transfer solution. The gel, sandwiched between a sheet of PVDF membrane and eight sheets of filter paper (Munktell, grade 1 F), was assembled into an LKB blotting apparatus and blotted for 75 min at 5 V in transfer solution. The PVDF membrane was stained with 0.25% (w/v) Coomassie brilliant blue R-250 in methanol:water:glacial acetic acid (5:5:1, by vol.) for 2 min and was destained with methanol:water:glacial acetic acid (4:5:1, by vol.) for 10 min (Brandt and von Holt 1986). Bands corresponding to the H1-like proteins e1, e2, e3, and e4 (Schlimme et al. 1993) were cut out and used for amino acid analysis.

Reversed-phase chromatography

HCl-extracted proteins were separated in a fast protein liquid chromatography (FPLC) system on a reversed-phase C1/C8 column (Pharmacia, ProRPC 5/10). Proteins were dissolved in 0.1% trifluoroacetic acid (TFA) and chromatographed at a constant flow of 0.5 ml/min using 0.1% TFA in water with increasing concentrations of acetonitrile. The eluted proteins were monitored at 214 nm, and peak fractions were collected separately and lyophilized.

Amino acid analysis

Acid-extracted, FPLC-purified or electroblotted histones were hydrolysed at 115° C for 22 h in 6 *M* HCl (Schaller et al. 1989). Amino acid analysis was carried out by reversed-phase high-performance liquid chromatography (HPLC) on a Nova-Pak C18 column in a Hewlett-Packard liquid chromatograph 1090 using the phenylisothiocyanate method (Bidlingmayer et al. 1984; Schaller et al. 1989). The molecular percentages (mol%) of the amino acids were calculated. The amino acids asparagine and glutamine were determined as their corresponding acids. Levels of cysteine and tryptophan were not determined.

The sums of the differences in the mol% values of the amino acids were calculated to determine the degree of similarity between the histone H1-like proteins e1–e4 of *T. b. brucei* (Schlimme et al. 1993) and the histone H1 of other organisms. In addition, the amino acid composition of each of the histone H1-like proteins was compared with the data reported for reference proteins in the SWISS PROT sequence data bank (24, release 8.0, February 1993; 28154 sequences) as described by Bender et al. (1992a). The 50 best similarity scores were considered by the computer program.

Preparation of soluble chromatin

Nuclear chromatin was digested with 0.2 units micrococcal nuclease (Sigma, N 3755) per 20 A_{260} at 30° C for 45 s. Chromatin was solubilized by nuclear lysis in a low-salt buffer containing 1 m*M* triethanolamine hydrochloride (TEACl, pH 7.4) and 0.2 m*M* disodiumethylenediaminetetraacetic acid (Na_2-EDTA). The conditions originally chosen were those previously described for higher eukaryotes (Thoma et al. 1979; Hecker and Gander 1985).

In later experiments, the method was modified to improve the preservation of the unstable chromatin of *T. b. brucei.* In the new preparation protocol, the period of nuclear lysis, during which the soluble chromatin was exposed to low salt concentrations, was reduced to 1 min. A buffer containing 5 m*M* TEACl (pH 7.4), 0.2 m*M* Na_2-EDTA, and 100 m*M* NaCl was immediately added

to the low-salt sample to make a final concentration of 10 m*M* NaCl. Nuclear debris was removed by centrifugation at 4000 *g* for 5 min. The supernatants containing the soluble chromatin were used directly for gradient analysis and electron microscopy or were dialysed overnight in a dialysis tube with a molecular-weight cut off of 300 kDa against 5 m*M* TEACl (pH 7.4), 0.2 m*M* Na_2-EDTA, and 10 m*M* NaCl to get rid of dissociated proteins.

Gradient centrifugation of soluble chromatin

Soluble chromatin was prepared as described above by either the original or the modified method. Sucrose-gradient centrifugation was performed according to the method of Thoma and Koller (1981) and Schlimme et al. (1993). The gradients were monitored at 254 nm and the bottom fractions containing the larger fragments of soluble chromatin were used for psoralen cross-linking or for electron microscopic analysis.

Psoralen cross-linking

Isolated nuclei were resuspended in 90 m*M* suspension buffer (pH 7.4; Thoma et al. 1979; Hecker et al. 1989). Large soluble-chromatin fragments were dialysed overnight against 5 m*M* TEACl (pH 7.4), 0.2 m*M* Na_2-EDTA, and 10 m*M* NaCl. Samples of nuclear suspension or of soluble chromatin containing 50 µg DNA were cross-linked under long-wavelength UV irradiation with 4,5′,8-trimethylpsoralen (Sigma, P 8399) in open petri dishes on ice for 6 h (Sogo et al. 1984). The DNA was purified as described by Sambrook et al. (1989), resuspended at a concentration of about 1 µg DNA/10 µl 2 m*M* Na_2-EDTA, 20 m*M* TRIS-HCl (pH 7.8), and stored at 4° C until used for electron microscopy.

Dissociation of chromatin-bound components

Soluble chromatin prepared according to the conditions adapted to *T. b. brucei* was used. Long fragments of soluble chromatin were separated from short fragments by centrifugation of 50 A_{260} soluble chromatin in 5%–25% (w/v) 5-ml isokinetic sucrose gradients containing 5 m*M* TEACl (pH 7.4), 0.2 m*M* Na_2-EDTA, and 10 m*M* NaCl. Centrifugation was performed for 1 h at 150000 *g* in a Kontron TST 55.5 swing-out rotor. Long fragments were dialysed (molecular weight cut-off, 8000 Da) overnight against 5 m*M* TEACl (pH 7.4), 0.2 m*M* Na_2-EDTA, and 10 m*M* NaCl containing 0.5 m*M* phenylmethylsulfonylfluoride (PMSF).

The volume of the sample was reduced to 2.5 ml with polyethylene glycol (molecular weight, 20000 Da) and the chromatin was centrifuged through 5.5%–28.5% (w/v) 17-ml isokinetic sucrose gradients containing 5 m*M* TEACl (pH 7.4), 0.2 m*M* Na_2-EDTA, and 500 m*M* NaCl for 15 h at 25000 *g* in a Kontron TST 28/17 swing-out rotor (Thoma and Koller 1981; Schlimme et al. 1993). The gradients were monitored at 254 nm and divided into 10 aliquots, which were dialysed against 0.5 m*M* PMSF in water. The samples were then lyophilized, and the proteins analysed by Triton acid-urea gel electrophoresis. Alternatively, the bottom fractions containing the larger fragments of soluble chromatin were used for electron microscopy.

Electron microscopy

The fractions with the large chromatin fragments were divided into four aliquots and dialysed against 5 m*M* TEACl (pH 7.4) and 0.2 m*M* Na_2-EDTA containing 0, 10, 40, or 100 m*M* NaCl, respectively, for 4 h. Then, the dialysis buffer was adjusted to 0.2% (v/v) with glutaraldehyde (Schlimme et al. 1993), and the samples were fixed for at least 15 h and prepared for electron microscopy as described by Thoma et al. (1979).

Psoralen-cross-linked DNA was denatured and spread onto bidistilled water by the benzyldimethyl-alkyl-ammonium chloride (BAC) method (Sogo et al. 1984) and then prepared for electron microscopy (Hecker et al. 1989).

Results

Two-dimensional gel electrophoresis

Proteins from isolated nuclei were extracted with 0.25 *N* HCl and separated in two-dimensional gels to see how many different variants or posttranslational modifications of the histones were present. The letters a, b, c, and d designate the core histones, and e1, e2, e3, and e4 designate a group of histone H1-like proteins.

Triton acid-urea/SDS-Tricine two-dimensional gel electrophoresis was chosen to separate the proteins according to their hydrophobicity and molecular weight (Fig. 1a). The sequence in the second dimension was histone a, b1, b2, c, e1, d, and e2–e4 according to the terminology introduced for histones separated on SDS-Tricine gels by Schlimme et al. (1993). The sequence in

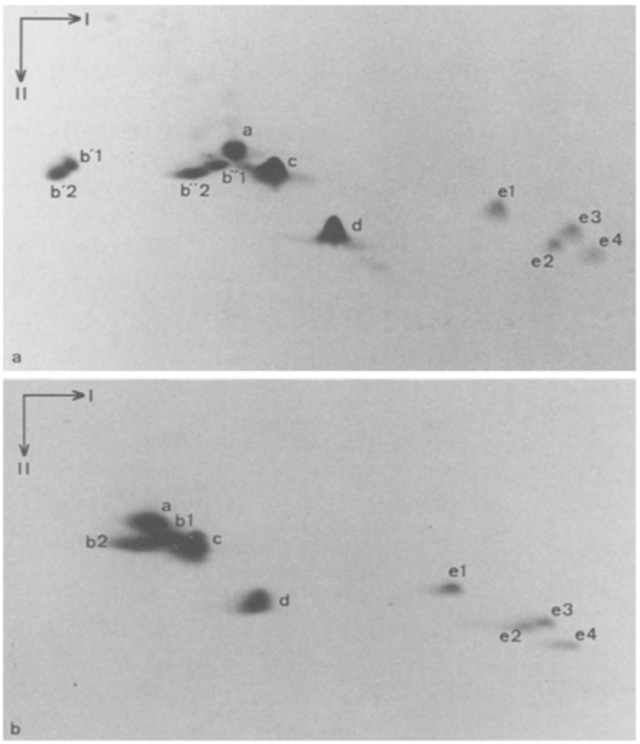

Fig. 1a, b. *Trypanosoma brucei brucei* histones analyzed by two-dimensional gel electrophoresis. HCl-extracted proteins from isolated nuclei were first separated **a** in a Triton acid-urea gel according to their hydrophobicity (*I*) or **b** in an acid-urea gel according to their charge (*I*). A strip of gel was then put on a linear SDS-Tricine gel and proteins were separated in the second dimension according to their molecular weights (*II*). Histones were separated into five main complexes. *a–d*, core histones; *e*, histone H1-like proteins. Variants and/or posttranslational modifications of the core histone b and of the histone H1-like proteins are indicated by *numbers*, (′), or (″)

the first dimension was histone b, a, c, d, and e. Two complexes of histone b with different hydrophobic properties were visible (b′ and b″). Each complex consisted of two spots of histone b with different molecular weights (b′1/b′2 and b″1/b″2). The histone H1-like proteins of the e complex were separated into four spots. The sequence in the first dimension was e1, e2, e3, and e4. The histone H1-like proteins e2, e3, and e4 had similar hydrophobic properties. The sequence of these proteins in the second dimension was e1, e3, e2, and e4. They migrated in the same region as the core histone d in the second dimension.

Acid-urea/SDS-Tricine two-dimensional gel electrophoresis was performed to separate the proteins according to their charge and molecular weight (Fig. 1b). Histone b was separated into two complexes with different charge properties and different molecular weights (b1 and b2). The proteins of the e complex were again separated into four spots. The sequence in both dimensions was similar to that observed in the Triton acid-urea/SDS-Tricine gel.

Fractionation of histone H1-like proteins and core histones

Histones were extracted from purified nuclei with 0.25 *N* HCl and were fractionated by FPLC reversed-phase chromatography. Bulk material was eluted in two major peaks (Fig. 2). The histone H1-like proteins were eluted in the range of 23%–26% acetonitrile. The core histones eluted at high acetonitrile concentrations. No improvement in the resolution of individual proteins resulted when the histone H1-like proteins were rechromatographed with a shallow water/acetonitrile gradient (results not shown).

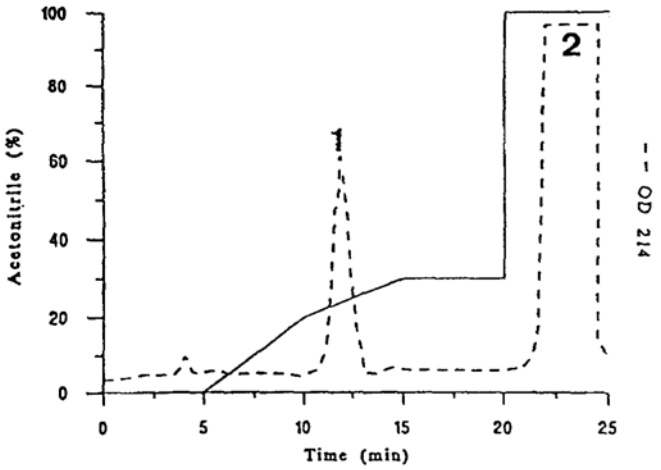

Fig. 2. Fractionation of histone H1-like proteins and core histones of *T. b. brucei* by FPLC reversed-phase chromatography. Proteins in 0.1% trifluoroacetic acid (TFA) were loaded onto a reversed-phase C1/C8 column. Solvents: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile. Gradient: 100% A, 5 min; 0–20% B, 5 min; 20%–30% B, 5 min; 30% B, 5 min; directly to 100% B, 5 min. Flow rate, 0.5 ml/min. *Dashed line*, eluted proteins monitored at 214 nm. Peak *1*, containing the histone H1-like proteins, and peak 2, containing the core histones, were isolated

Amino acid composition

Amino acid analysis of proteins extracted with 0.25 *N* HCl from isolated nuclei as well as of the FPLC-purified total core histones (fraction 2) revealed a high content of lysine, arginine, glutamic acid, and alanine (Table 1). Ratios of basic to acidic residues of 1.44–1.70 were found. The ratios of lysine to arginine ranged between 1.0 and 1.16. Considerable differences existed in the amounts of lysine, glycine, threonine, alanine, leucine, phenylalanine, and methionine.

The amino acid analysis of proteins extracted with 5% PCA from isolated nuclei or of FPLC-purified total histone H1-like proteins (fraction 1) as well as of individual histone H1-like proteins e1–e4 yielded high mol% values for lysine, alanine, valine, and proline and a low content of arginine, aspartic acid, glutamic acid, tyrosine, leucine, isoleucine, phenylalanine, and methionine (Table 1). High ratios of basic to acidic amino acids as well as of lysine to arginine were found. The amino acid composition of proteins extracted with 5% PCA from isolated nuclei differed from that of the FPLC-purified total histone H1-like proteins in the amounts of the amino acids lysine, histidine, glycine, threonine, tyrosine, leucine, and methionine. No histidine was detected in the individual histone H1-like proteins e1–e4, although small amounts were present in FPLC-purified total histone H1-like proteins.

The sum of the differences in the mol% values of individual amino acids can be used to assess the degree of similarity between histone H1-like proteins e1–e4 of *Trypanosoma brucei brucei* and the histone H1 of other organisms (Schaller, personal communication; Fig. 3). Thus, all histone H1-like proteins of *T. b. brucei* appear to be closely related to H1 from *T. cruzi* (Toro et al. 1993) and *Oxytricha spp.* (Caplan 1975). The histone H1-like proteins e1 and e2 are more closely related to calf thymus histone H1 than are proteins e3 and e4. All histone H1-like proteins of *T. b. brucei* are less closely related to H1 from *Crithidia fasciculata* (Duschak and Cazzulo 1990), *Neurospora crassa* (Goff 1976), and *Tetrahymena pyriformis* (Gorovsky et al. 1974).

When the amino acid composition of the histone H1-like proteins of *T. b. brucei* was compared with that of proteins in the SWISS PROT sequence data bank, high scores of similarity to histone H1 were found. The percentage of histone H1 among the 50 best similarity scores was 72% for proteins e1, e2, and e4 and 88% for protein e3 when only the relative amounts of individual amino acids were considered by the computer program. When the absolute numbers of each species of amino acid per histone molecule were considered, the values were 88% for e1, 90% for e2, 98% for e3, and 84% for e4.

Table 1. Amino acid composition of different preparations of histones of *Trypanosoma brucei brucei* and of histone H1 of calf thymus and of various lower eukaryotes. All values are expressed in mol%. aa, amino acid; HCl, proteins extracted with 0.25 *N* HCl from isolated nuclei; core, FPLC-purified total core histones; PCA, proteins extracted with 5% PCA from isolated nuclei; H1-l. p., FPLC-purified total histone H1-like proteins; e1–e4, individual histone H1-like proteins of *T. b. brucei*; c. t., calf thymus (Panyim et al. 1971); C. f., *Crithidia fasciculata* (Duschak and Cazzulo 1990); N. c., *Neurospora crassa* (Goff 1976); O. spp., *Oxytricha* spp. (Caplan 1975); T. p., *Tetrahymena pyriformis* (Gorovsky et al. 1974); T. c., *T. cruzi* (Toro et al. 1993); K:R, ratio of lysine to arginine; b:a, ratio of basic to acidic residues; n.d. not detected

aa	HCl	core	PCA	H1-l. p.	e1	e2	e3	e4	c. t.	C. f.	N. c.	O. spp.	T. p.	T. c.
Lys	12,8	10,5	15,8	24,5	20,6	22,4	27,8	20,2	27,3	15,4	16,8	31,6	29,9	32,4
Arg	11,0	10,4	1,2	1,4	1,5	1,4	1,4	1,2	2,1	3,6	3,4	3,4	2,4	2,0
His	1,6	1,5	1,8	0,3	0,0	0,0	0,0	0,0	0,0	2,1	1,5	0,5	2,3	1,3
Asp	5,7	5,6	2,3	3,3	2,7	3,9	3,4	3,1	2,6	5,3	6,2	1,9	9,9	3,7
Glu	9,8	9,9	2,8	2,0	2,3	3,0	2,0	2,2	4,7	8,7	8,5	1,2	7,3	2,6
Gly	6,7	8,8	6,4	3,3	3,9	3,2	4,2	2,4	6,7	16,7	5,8	2,8	3,5	4,3
Ser	6,6	6,2	3,0	3,7	3,0	3,6	3,8	3,7	6,9	6,7	5,7	6,9	7,5	5,9
Thr	7,1	6,0	5,2	3,0	3,5	2,5	2,9	3,6	6,0	3,3	6,3	4,1	8,6	1,8
Tyr	2,3	2,1	0,0	0,7	1,6	1,7	0,5	0,6	0,6	1,4	1,5	0,2	0,5	0,7
Ala	13,8	11,6	33,1	34,1	36,5	36,3	31,2	35,8	23,4	18,9	21,1	29,2	14,0	26,5
Val	6,5	7,2	9,1	11,4	14,0	10,2	10,1	13,9	6,0	3,4	4,9	8,1	3,9	4,8
Leu	6,8	8,6	2,4	0,8	1,7	0,8	0,7	1,0	3,9	3,5	4,8	0,3	1,7	1,5
Ile	2,8	3,3	0,9	0,3	0,7	0,7	0,3	0,8	0,8	1,9	2,0	4,3	2,3	0,8
Pro	3,7	3,2	12,1	10,1	7,5	9,6	11,6	11,2	8,4	6,2	7,6	5,1	6,1	11,0
Phe	2,1	3,4	0,6	0,3	0,7	0,7	0,3	0,3	0,6	2,0	1,8	0,2	0,4	0,5
Met	0,8	1,6	3,5	0,4	0,0	0,0	0,0	0,0	trace	1,1	1,2	0,0	trace	n.d.
K : R	1,16	1,00	13,70	17,35	13,44	15,85	19,28	16,58	13,00	4,28	4,94	9,29	12,46	16,18
b : a	1,70	1,44	3,68	4,90	4,43	3,43	5,43	4,05	4,03	1,51	1,48	11,45	2,01	5,65

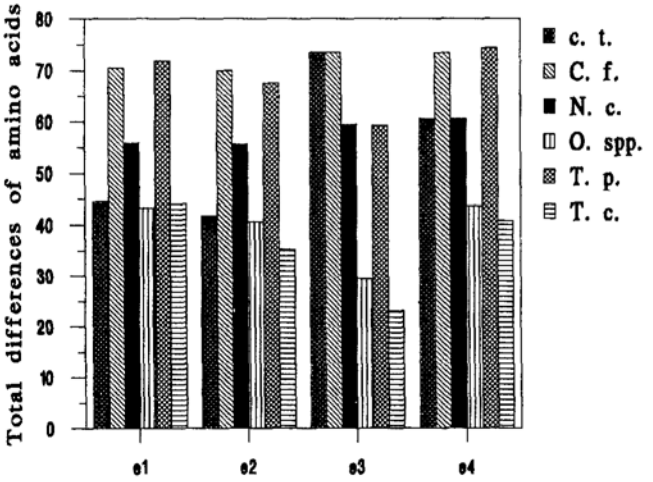

Fig. 3. Comparison of the sum of differences in mol% of individual amino acids between histone H1-like proteins e1–e4 of *T. b. brucei* and H1 of calf thymus (*c. t.*; Panyim et al. 1971); *Crithidia fasciculata* (*C. f.*; Duschak and Cazzulo 1990); *Neurospora crassa* (*N. c.*; Goff 1976); *Oxytricha* spp. (*O. spp.*; Caplan 1975); *Tetrahymena pyriformis* (*T. p.*; Gorovsky et al. 1974), and *T. cruzi* (*T. c.*; Toro et al. 1993). The lower the value of the difference, the higher the degree of similarity between two proteins

Effect of the preparation method on the stability of the soluble chromatin

Chromatin of procyclic *T. b. brucei* has previously been prepared according to conditions adapted for higher eukaryotes (Thoma et al. 1979; Hecker and Gander 1985). Studies on the ultrastructure of the chromatin revealed poor conservation of the nucleosome filaments, and no condensation was observed in the presence of 10–100 m*M* salt. In an attempt to preserve its structure better, soluble chromatin was prepared according to a modified protocol. In the new procedure, the periods of nuclear lysis and of exposure of soluble chromatin to low-salt conditions were reduced from 0.5 h to 1 min. The ultrastructure of the chromatin was investigated by electron microscopy and compared with that of chromatin prepared according to standard conditions.

Soluble chromatin of *T. b. brucei* procyclic culture forms was centrifuged through sucrose gradients containing 10 m*M* NaCl and dialysed against concentrations of 0, 10, 40, or 100 m*M* NaCl. Soluble chromatin prepared under conditions adapted to procyclic *T. b. brucei* was composed of regularly spaced nucleosome filaments and showed some condensation at increasing ionic strength (Fig. 4e–h). Nucleosome filaments of soluble chromatin prepared under standard conditions were irregularly spaced and barely condensed at increasing concentrations of NaCl (Fig. 4a–d). In the absence of salt, displacement of nucleosomes occurs (Fig. 4a, e), but the phenomenon is less pronounced than in chromatin prepared according to the former standard conditions.

Isolated nuclei were subjected to psoralen cross-linking at 90 m*M* NaCl (pH 7.4). The isolated and spread DNA appeared in the form of single-stranded bubbles separated by short stretches of double-stranded DNA (Fig. 5a). Soluble chromatin was centrifuged through sucrose gradients containing 10 m*M* NaCl and cross-linked with psoralen under low-salt conditions (pH 7.4). Regular arrays of single-stranded bubbles separated by short stretches of double-stranded DNA were seen in the DNA of soluble chromatin that had been prepared under conditions adapted to procyclic *T. b. brucei* (Fig. 5c). In contrast, the DNA of soluble chromatin prepared under the former standard conditions showed

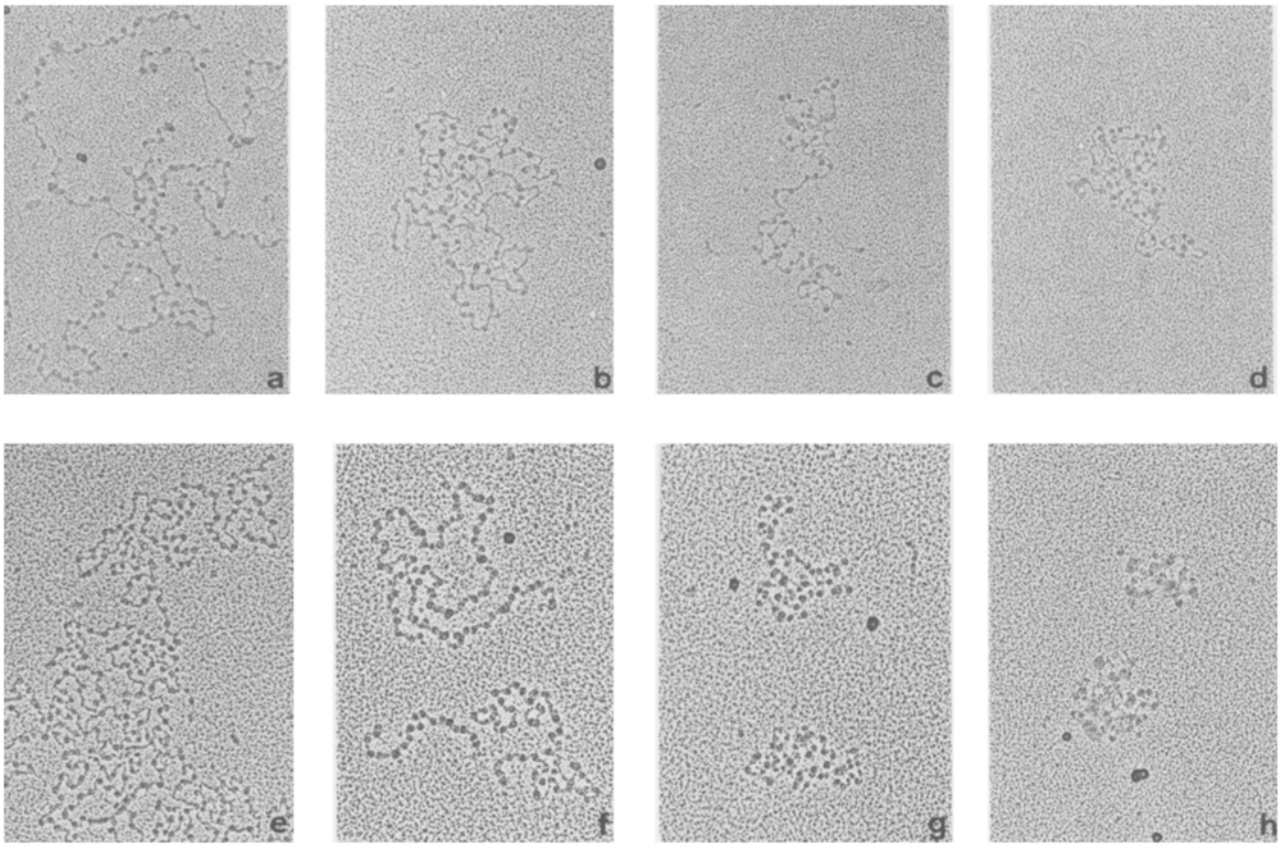

Fig. 4a–h. Electron microscopy of chromatin of *T. b. brucei* prepared under different conditions. Soluble chromatin was dialysed against 0, 10, 40, or 100 m*M* NaCl, fixed and prepared for electron microscopy. Soluble chromatin prepared according to standard conditions adapted to higher eukaryotes showed irregularly spaced nucleosomes and only barely condensed at increasing ionic strength (**a–d**). Soluble chromatin prepared under conditions adapted to *T. b. brucei* was composed of regularly spaced nucleosome filaments and condensed at increasing concentrations of NaCl (**e–h**). **a, e**: 0 m*M* NaCl. **b, f**: 10 m*M* NaCl. **c, g**: 40 m*M* NaCl. **d, h**: 100 m*M* NaCl. Magnification, ×60000

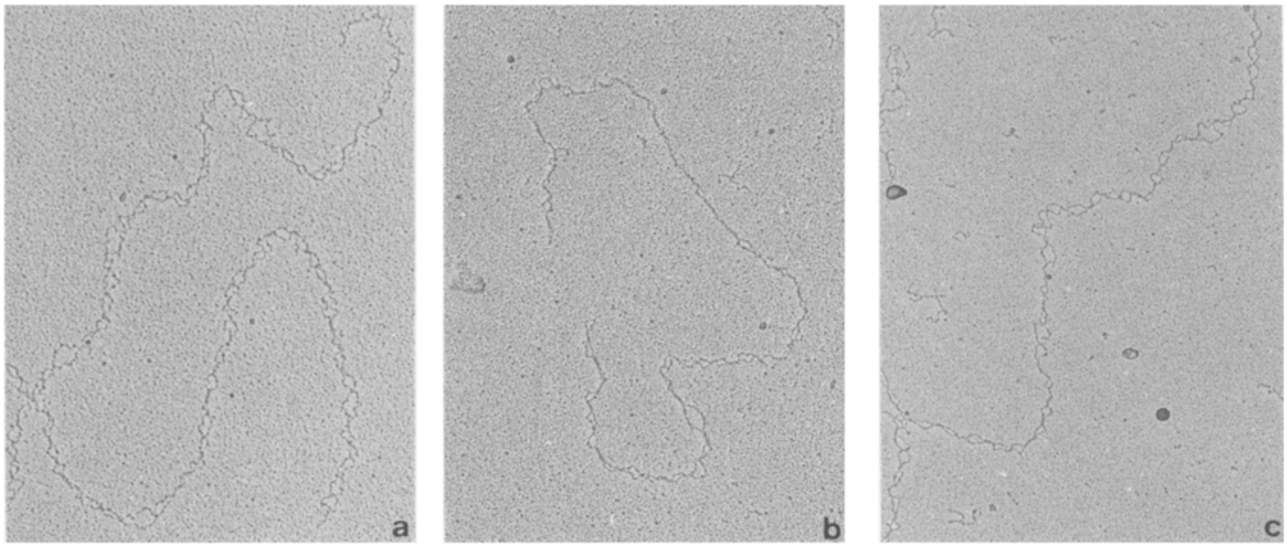

Fig. 5a–c. DNA structure of nuclear chromatin of *T. b. brucei* after psoralen cross-linking, DNA purification, denaturation, and spreading. Chromatin of isolated nuclei cross-linked at 90 m*M* salt (pH 7.4) showed regular arrays of single-stranded bubbles (**a**). Soluble chromatin prepared under different conditions was fractionated in a 10 m*M* NaCl-containing sucrose gradient and cross-linked at 10 m*M* NaCl. Soluble chromatin prepared using standard conditions adapted to higher eukaryotes showed no regular arrays of single-stranded bubbles (**b**). Soluble chromatin prepared under conditions adapted to *T. b. brucei* consisted of regular arrays of single-stranded bubbles (**c**). Magnification, ×60000

single-stranded bubbles separated by variable stretches of double-stranded DNA (Fig. 5b).

Samples of soluble chromatin prepared by both methods were dialysed overnight with a molecular-weight cut-off of 300000 Da against 15 m*M* salt. When the proteins remaining bound to the chromatin were lyophilized and analyzed in a Triton acid-urea gel, only soluble chromatin prepared according to the new conditions contained the full set of histones (Fig. 6, lanes 3 and 1). Soluble chromatin prepared according to the conditions used previously contained no histone H1-like proteins of the e region (Fig. 6, lane 2).

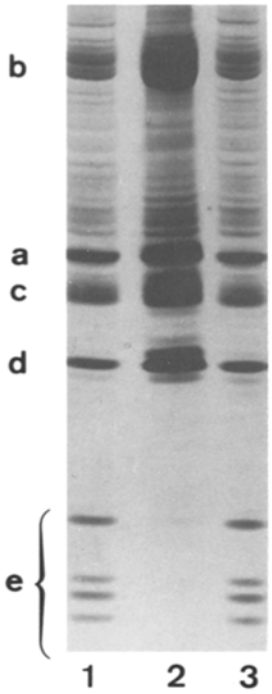

Fig. 6. Analysis of histones of *T. b. brucei* in a Triton acid-urea gel. HCl-extracted proteins from isolated nuclei (*lane 1*). *a–d*, core histones; *e*, histone H1-like proteins. Soluble chromatin was dialysed overnight with a molecular weight cut-off of 300000 Da against 5 m*M* TEACl, 0.2 m*M* Na_2-EDTA, and 10 m*M* NaCl (pH 7.4). Proteins remaining in the dialysis bag were separated according to their hydrophobicity. Chromatin prepared according to standard conditions adapted to higher eukaryotes contained no histone H1-like proteins of the e region (*lane 2*). Chromatin prepared according to conditions adapted to *T. b. brucei* contained the full set of histones (*lane 3*)

Dissociation of chromatin-bound components

Long nucleosome filaments from soluble chromatin that had been prepared according to the modified method were centrifuged through an isokinetic sucrose gradient containing 500 m*M* NaCl, which is known to dissociate histone H1 completely from the chromatin of higher eukaryotes. The nucleosome filaments that resulted showed irregularly spaced nucleosomes, and a salt-dependent condensation could barely be observed (Fig. 7a–d). The fine structure of the nucleosome filaments was similar to that of nucleosome filaments prepared according to the method used for higher eukaryotes (Fig. 4a–d). At 40 and 100 m*M* NaCl, a slight aggregation of the nucleosomes was visible (Fig. 7c, d).

Analysis of the proteins of individual fractions of the gradient in a Triton acid-urea gel revealed that the "top" fractions 7–9 contained the bulk of the histone H1-like proteins e1–e4 (Fig. 8, lanes 8–10). No core histones could be detected in fraction 8 or 9 (Fig. 8, lanes 9 and 10). The "bottom" fractions 1–6 contained the core histones a–d. No band was seen migrating in the e area (Fig. 8, lanes 2–7).

Discussion

The presence of histone H1 in the chromatin of *Trypanosoma brucei brucei* has been questioned in various gel systems, since no protein with its characteristic electrophoretic mobility has been found. Analysis of the chromatin by electron microscopy provided further evidence for a histone H1-depleted chromatin fiber, since no condensation could be observed in response to increasing salt concentrations (Hecker and Gander 1985; Hecker et al. 1989; Bender et al. 1992a–d). However, recent experiments using modified gel systems for the analysis of histones have shown the presence of histone H1-like proteins in *T. b. brucei* (Schlimme et al. 1993). Nevertheless, these authors could not show a salt-dependent condensation of the chromatin.

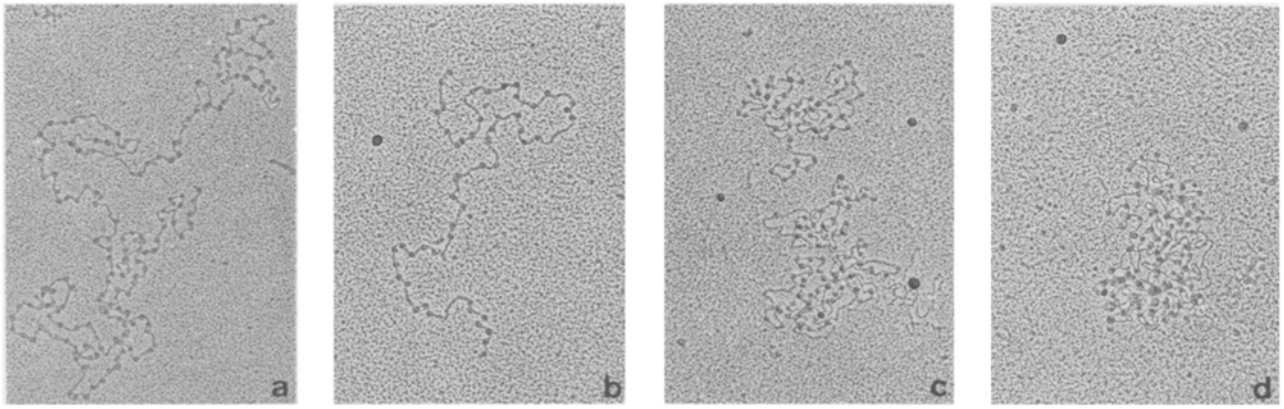

Fig. 7a–d. Long nucleosome filaments of soluble chromatin prepared under conditions adapted to *T. b. brucei*, centrifuged through sucrose gradients containing 500 m*M* NaCl, dialysed against increasing concentrations of salt, fixed and prepared for electron microscopy. The nucleosomes were irregularly spaced and the chromatin barely condensed at increasing concentrations of salt. **a** 0 m*M* NaCl. **b** 10 m*M* NaCl. **c** 40 m*M* NaCl. **d** 100 m*M* NaCl. Magnification, ×60000

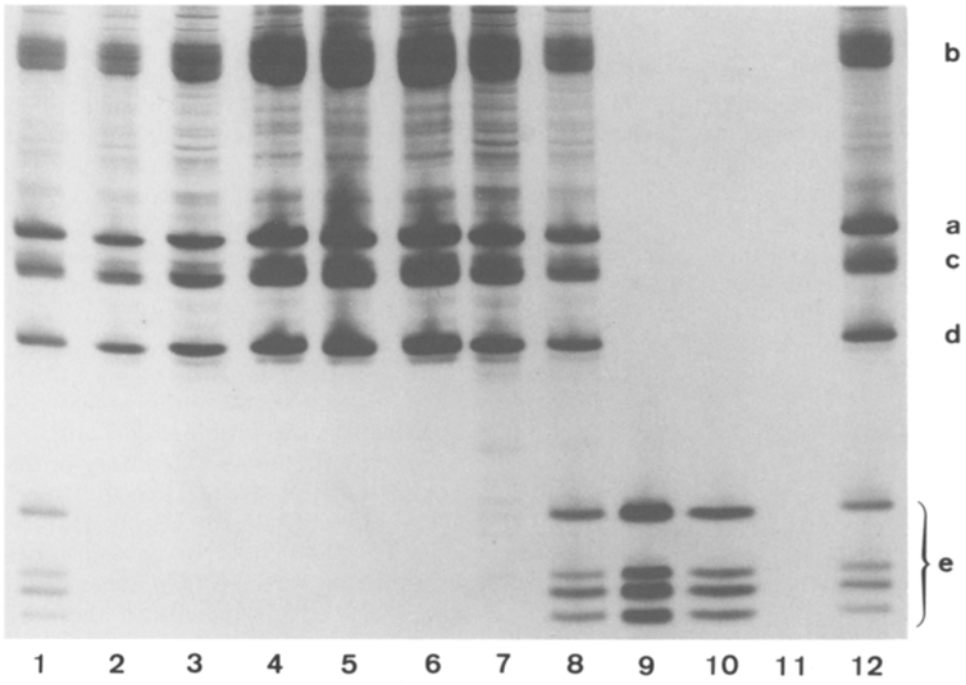

Fig. 8. Long nucleosome filaments of soluble chromatin prepared under conditions adapted to *T. b. brucei* and fractionated by sucrose-gradient centrifugation in the presence of 500 m*M* NaCl, and proteins of individual fractions analyzed by Triton acid-urea gel electrophoresis. *Lanes 1, 12*, marker: unfractionated soluble chromatin; *lanes 2–11*, fraction 1 (*bottom*) through fraction 10 (*top*). *a–d*, core histones; *e*, histone H1-like proteins. The histone H1-like proteins of the e region dissociated from the chromatin and appeared in the *top* fractions of the gradient

In the present study, when soluble chromatin of *T. b. brucei* procyclic culture forms was prepared according to standard conditions adapted to higher eukaryotes (Thoma et al. 1979; Hecker and Gander 1985), no condensation in response to salt was observed. The nucleosome filaments were poorly preserved and individual nucleosomes dissociated from the chromatin at 0 m*M* NaCl. Since this behavior is characteristic of chromatin in which histone H1 has been depleted (Thoma et al. 1979), we decided to find out whether the histone H1-like proteins had been lost from *T. b. brucei* chromatin during preparation. Therefore, soluble chromatin was dialysed using a membrane with a large molecular-weight cut off, and the proteins remaining in the dialysis bag were analyzed by Triton acid-urea polyacrylamide gel electrophoresis. The observed absence of the histone H1-like proteins from the sample indicated that these proteins must have dissociated from the chromatin because their interaction with the DNA was weak under low-salt conditions. The absence of the histone H1-like proteins in the chromatin explained both its inability to condense and the irregular spacing of the nucleosomes on the DNA (Stein and Bina 1984) as well as the observation that no regular array of single-stranded bubbles was seen in the DNA of psoralen-cross-linked soluble chromatin.

Our ultrastructural results were comparable with the observations made by Hecker and Gander (1985), Hecker et al. (1989), and Schlimme et al. (1993) using chromatin prepared under the same conditions and adapted to the chromatin of higher eukaryotes. Our results indicated that the soluble chromatin of *T. b. brucei* used in previous studies had most probably also been depleted of histone H1.

Since histone H1-like proteins are known to be present in *T. b. brucei* chromatin, we investigated whether the isolation conditions could be modified such that these proteins would be retained. Since it seemed possible that the instability of the chromatin was due to the long time the samples spent in low-salt solutions during the preparation, the periods of lysis of the nuclei and of exposure of the soluble chromatin to low-salt buffer were reduced from 30 to 1 min.

Soluble chromatin prepared according to the new conditions adapted to *T. b. brucei* indeed contained the full set of histones, including histone H1-like proteins. When its ultrastructure was studied, it was found to be composed of regularly spaced nucleosome filaments and to show salt-dependent condensation. However, at 0 m*M* salt, some of the nucleosomes dissociated from the chromatin. This observation lends support to the suggestion that there is a time-dependent instability of the chromatin under low-salt conditions. When soluble chromatin was cross-linked with psoralen at 10 m*M* NaCl (pH 7.4), regular arrays of single-stranded bubbles separated by short stretches of double-stranded DNA were visible. The cross-linkage pattern of the DNA was comparable with that of the DNA cross-linked at 90 m*M* salt in isolated nuclei (Hecker et al. 1989; present results). This indicated that the DNA-protein interactions in the nucleosomes had been preserved.

By the use of FPLC reversed-phase chromatography we were capable of separating the histone H1-like proteins from the core histones, which bound more strongly to the reversed-phase column. The amino acid composition of the fraction containing the total histone H1-like proteins was rich in lysine, alanine, and proline and contained only a small proportion of arginine, which is typical for histone H1. The ratios of basic to acidic amino acids and lysine to arginine were similar to those of H1 from calf thymus (Panyim et al. 1971) and *T. cruzi*

(Toro et al. 1993). The amino acid composition of FPLC-purified total histone H1-like proteins showed some differences from that of the proteins extracted with 5% PCA, which might be explained by the presence of nonhistone proteins in the 5% PCA extract (Sanders 1977). The amino acid composition of FPLC-purified bulk core histones was comparable with that of proteins extracted with 0.25 *N* HCl from isolated nuclei. This could be explained by the observation that core histones are the most prominent basic proteins present in the nuclei (Johns 1967). Differences in the proportions of individual amino acids could be explained by the presence of histone H1-like proteins in the HCl-extracted proteins. Gurley et al. (1990) have postulated that the retention of the histones in the reversed-phase column is determined by the number of strongly hydrophobic ion pairs between trifluoroacetic acid (TFA) and arginine. The different content of arginine could explain the different binding properties of the histone H1-like proteins and the core histones of *T. b. brucei* to the column.

To verify that all of the four proteins eluted in fraction 1 were histone H1-like, they were separated in Triton acid-urea gels and blotted onto polyvinylidenedifluoride (PVDF) membranes (Brandt and von Holt 1986). Due to the high resolution in the Triton acid-urea gel (Toro and Galanti 1988), individual stained proteins could be excised from the membrane and used for amino acid analysis. The high frequency of similarity scores for H1 indicated that all four of the proteins e1–e4 showed an amino acid composition typical for histone H1 (reviewed by van Holde 1989). Similarities in the amino acid composition, in the ratio of basic to acidic amino acids, and in the ratio of lysine to arginine between the H1-like proteins of *T. b. brucei* and the histone H1 of calf thymus (Panyim et al. 1971), of *T. cruzi* (Toro et al. 1993) or of *Oxytricha* spp. (Caplan 1975) also indicated that these proteins are biochemically related. Disparities in the content of amino acids of individual histone H1-like proteins (e1–e4) of *T. b. brucei* indicated the presence of variants, which was in accordance with the heterogeneity seen in two-dimensional gels as well as with the reported variability of H1 (reviewed by van Holde 1989). Differences in the amino acid composition among individual histone H1-like proteins as well as between them and the histone H1 from other lower eukaryotes such as *T. cruzi* (Toro et al. 1993), *Crithidia fasciculata* (Duschak and Cazzulo 1990), *Neurospora crassa* (Goff 1976), *Oxytricha* spp. (Caplan 1975), and *Tetrahymena pyriformis* (Gorovsky et al. 1974) indicated the unique character of the histone H1-like proteins of *T. b. brucei*.

The investigation of the interaction of the histone H1-like proteins with the DNA by modifying the binding forces with salt indicated that the histone H1-like proteins of *T. b. brucei* function similarly to histone H1 in the setting of regularly spaced nucleosomes and in the condensation of the chromatin (Thoma et al. 1979; Stein and Bina 1984; Thomas 1984; Widom 1989). At 500 m*M* NaCl, a selective dissociation of the histone H1-like proteins from the DNA took place. The resultant nucleosome filaments appeared as a randomly bent string of irregularly arranged nucleosomes that barely condensed at increasing ionic strength. The fine structure of the chromatin was comparable with that of chromatin prepared at conditions not especially adapted to *T. b. brucei*, where the histone H1-like proteins are lost, as well as with that of the chromatin of higher eukaryotes depleted of histone H1 (Thoma and Koller 1981; Thoma et al. 1979).

The characteristics of the histone H1-like proteins of *T. b. brucei* can be summarized as follows from the results of the present investigation and from those of Schlimme et al. (1993):

1. Depending on the stage of the life cycle, the histone H1-like proteins of *T. b. brucei* are present in three or four variants and/or modifications (e1–e4).
2. They can be extracted from isolated nuclei or soluble chromatin with 0.25 *N* HCl, 5% perchloric acid (PCA), or 500 m*M* NaCl.
3. They are not extractable from isolated nuclei with 350 m*M* NaCl.
4. In sodium dodecyl sulfate (SDS)-Tricine gels they run in the region of the core histones, and in Triton acid-urea gels and acid-urea gels they run in front of the core histones. These migration patterns are different from those of histone H1 of calf thymus but are similar to those of the H1-like proteins of other protozoa such as *T. cruzi* (Toro and Galanti 1988, 1990), of *C. fasciculata* (Duschak and Cazzulo 1990), and of *Oxytricha* spp. (Caplan 1975).
5. They show similar staining and destaining behavior on Triton acid-urea gels with bromophenol blue and 40% *n*-propanol as compared with H1 from calf thymus.
6. Their amino acid composition and their binding properties to a reversed-phase column are similar to those of other histones H1.
7. They possess an extremely weak interaction with the chromatin at 0 m*M* NaCl and are therefore lost unless the chromatin is prepared using a very short period of exposure to low-salt conditions.
8. They contribute to the regular spacing of nucleosomes on the DNA.
9. They contribute to salt-dependent condensation of the chromatin.

All these properties indicate the histone H1 character of the histone H1-like proteins of *T. b. brucei*. However, the histone H1-like proteins of *T. b. brucei* do not appear to be identical to the histone H1 of higher eukaryotes as reflected by their weak interaction with DNA under low-salt conditions and by the observation that the chromatin of the protozoan parasite does not condense into 30 nm fibers, even after the use of an adapted preparation method.

The identification of four variants and/or posttranslational modifications of the core histone b demonstrated the heterogeneity of this histone. A similar heterogeneity is also found in histone H2A, the higher-eukaryotic counterpart of histone b (van Holde 1989; Bender et al. 1992a). The slight aggregation of nucleosome filaments that occurred even in chromatin depleted of histone H1-like proteins could be attributed to the core histones. It has been suggested that the N-terminal domains of

the core histones are involved in binding the DNA to the core particle or, alternatively, in the folding of the chromatin (Allan et al. 1982; Ausio et al. 1989; Widom 1989).

Condensation of the chromatin of *T. b. brucei* prepared under our adapted conditions did not reach the 30 nm fiber level. Therefore, it must differ from that of higher eukaryotes, which suggests that there is an alternative compaction pattern of the genetic information in the nucleus of the protozoan parasite. This difference might well be explained by the biochemically different variants and/or modifications of the histone H1-like proteins in *T. b. brucei*. In addition, the properties of the core histones may also influence the compaction pattern (Ausio et al. 1989); differences in the composition of these histones in *T. b. brucei* and in higher eukaryotes have also been found (Bender et al. 1992a–d), and variants and/or posttranslational modifications of the core histone b were identified by our study.

The differences in the level of condensation of the chromatin of *T. b. brucei* and that of higher eukaryotes is probably not an artifact of preparation, because in thin sections of nuclei of rat liver and *T. b. brucei* the chromatin fibers also differ in thickness (Hecker and Gander 1985). Furthermore, Vickerman and Preston (1970) have shown that no chromosomes can be visualized in dividing cells of trypanosomes.

Novel genetic and biochemical approaches have established that specific chromatin structures have a major impact on the transcription process. Precise alterations in histone sequence, nucleosome structure, and folding of the chromatin fiber influence both the activation and the repression of genes. Disparities found between histones of the mammalian host and those of the trypanosome parasite not only suggest that there are differences in the mechanisms regulating gene expression (Wolffe 1990, 1992; Zlatanova 1990; Felsenfeld 1992; Grunstein 1992) but could also be used to suggest possible targets for trypanocidal drugs.

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