Volume 257, number 2, 233–237 FEB 07759 November 1989

The histones of *Caenorhabditis elegans:* no evidence of stage-specific isoforms

An overview

Jacques R. Vanfleteren, Stefaan M. Van Bun† and Jozef J. Van Beeumen†

Laboratorium voor Morfologie en Systematiek der Dieren and †*Laboratorium voor Microbiologie en Microbiële Genetica, Rijksuniversiteit Gent, Ledeganckstraat 35, B-9000 Gent, Belgium*

Received 18 August 1989

The nematode *Caenorhabditis elegans* expresses one species of H2A and one species of H4 molecules, at least two species of H1 (H1.1, H1.2), two species of H2B (H2B.1, H2B.2) and 2–4 species of H3 (H3.1 and H3.3 and an unassigned Ile/Leu microheterogeneity in H3). The study of their primary structures has been completed now and all of them, with the exception of the Ile/Leu microheterogeneity in H3, have been assigned to protein spots on two-dimensional gels. One spot, previously designated H3.2, probably represents C-terminally cleaved H3.1. The relative abundance of the isohistones was essentially the same when derived from either eggs, gravid adults or postreproductive, senescent worms. The degree of post-translational modification, however, particularly acetylation of H2A, H2B and H3 histone species, was reduced at old age.

Amino acid sequence; Histone isoform; (*Caenorhabditis elegans*)

1. INTRODUCTION

Over the last decade, it has been shown that the major histone classes are frequently composed of a number of polypeptides that differ slightly in their primary structures. They are called isohistones, isoforms, subtypes, variants, isoprotein species, and they are usually designated by a number which is separated from the major class designated by a period, e.g. H1.1, H1.2, ... These variants may have no specific function, e.g. the H2A and H2B variants of yeast can replace each other [1,2]. On the contrary, in sea urchin, the expression of isohistones is strictly developmentally regulated and the structural properties of the chromatin vary with different isohistone composition [3]. Mammalian cells express three H3 variants, of which H3.1 and H3.2 are the more abundant isoforms, whereas H3.3 is a minor variant. However, in G1 and G2 cells and also in quiescent cells, only H3.3 is synthesized, the expression of H3.1 and H3.2 being fully repressed [4].

We were primarily interested in learning more about the possible differential expression of histone variants in the course of the life cycle of the nematode *Caenorhabditis elegans*. We have shown that a few nuclear non-histone proteins accumulate with age [5] and we wanted to know whether histones are involved in the onset and/or propagation of the aging process in *C. elegans*.

The primary structures of the major histone fractions from *C. elegans* have been published previously [6–10]. In the present study we will assign the various isohistone sequences to the protein spots seen on two-dimensional gels and examine whether alterations occur in the histone subtypes of *C. elegans* and their post-translational modification as a function of development and age.

Correspondence address: J.R. Vanfleteren, Laboratorium voor Morfologie en Systematiek der Dieren, Rijksuniversiteit Gent, Ledeganckstraat 35, B-9000 Gent, Belgium

Abbreviations: AUC, acetic acid/urea/cetyltrimethylammonium bromide; AUT, acetic acid/urea/Triton X-100; C, percentage concentration of bisacrylamide to the total concentration T; HPTLC, high-performance thin-layer chromatography; FUdR, fluorouracil deoxyribose; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PTH, phenylthiohydantoin derivative(s) of amino acid(s); T, total percentage concentration of acrylamide and bisacrylamide

2. EXPERIMENTAL

2.1. *Nematode culture*

The H2B subtypes were purified from unfractionated H2B that had been prepared from *Caenorhabditis elegans* strain DR27 *daf-17(m27)*. The cultivation of this strain was described at length earlier [6]. The characterization of H3.2 and the gel electrophoretic analyses of histones prepared from age-synchronized worms was done using the wild type Bristol (N2) strain of *C. elegans*. The worms were grown axenically as described previously [5,11]. The procedure for age synchronization was also described earlier [5]. Young gravid worms were between 5 and 10 days old. Senescent, post-reproductive worms, about 15–20 days old, were harvested 10 days after adding

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

50 μM FudR to exponentially growing cultures to block any further reproduction. Viable eggs were prepared as described by Johnson and Hirsch [12]. The eggs released were subsequently collected by centrifugation at 3000 × g for 10 min and washed extensively with S buffer (0.1 M NaCl, 0.05 M potassium phosphate buffer, pH 6.0). Cleaned nematodes or eggs were suspended in twice the volume of 1.7 M sucrose, 0.5% (v/v) Nonidet P-40, 5 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.4, immediately dripped in liquid nitrogen and stored at −196°C until use.

2.2. *Isolation and identification of H2B isoforms*

The H2B subtypes were separated on 2 preparative AUT gels (16 × 16 × 0.15 cm), loaded with approx. 4 mg of pure H2B [6] and run at 18 mA constant current (9 mA/gel). The gels were stained with 0.1% Coomassie brilliant blue R-250 in methanol/acetic acid/water (5:1:5) containing 1% (v/v) 2-mercaptoethanol and destained in 7% acetic acid, 20% ethanol, 1% 2-mercaptoethanol until the bands became clearly visible (fig.1a). The protein bands containing H2B.1 and H2B.2 were sliced out and protein was recovered as described previously [8]. Both H2B subtypes were dissolved in 50 μl ammonium acetate buffer (pH 4.0) and digested with 25 μg *Staphylococcus aureus* V8 proteinase (EC 3.4.21.19; Miles) overnight at 37°C. The resulting peptides were separated by preparative HPTLC and prepared for sequencing as described previously [6].

2.3. *Isolation of histone fraction H3.2*

Since we had reason to suspect that H3.2 is derived from H3.1 by degradation [8], a very quick extraction procedure was devised, aimed to minimize possible in vitro degradation of H3 at the expense of obtaining less pure histone. Approximately 100 g of frozen nematode tissue was crushed in a 1 l stainless steel cup (precooled at −25°C) of a Waring blendor for 1–2 min. After thawing, the concentration of sucrose was lowered to 0.25 M and PMSF and aprotinin were added at 1 mM and 0.1 mg/l, respectively. The homogenate was blended at full speed for 2 min more and centrifuged at 6000 × g for 5 min. The sediment was washed once with 0.14 M NaCl, 50 mM Tris-HCl, pH 7.4 and 1 mM PMSF was freshly added. Chromatin was extracted by homogenization of the pellet in 2 M NaCl, 10 mM Tris-HCl, pH 7.4 for 30 min (Virtis homogenizer, low speed) in the cold, and clarified by centrifugation at 10000 × g for 10 min. HCl, 2-mercaptoethanol and thiodiethanol were added to the supernatant at 0.5 M and 1% (v/v) final concentrations, respectively. The mixture was stirred vigorously overnight at 4°C. The histones were next clarified by centrifugation at 100000 × g for 20 min and precipitated by the addition of Reinecke salt [13]. The precipitate was collected by centrifugation at 3000 × g for 20 min in a swing-out rotor and washed with a saturated solution of Reinecke salt in 50 mM Tris-HCl, pH 6.2. Many non-histone proteins that were not highly charged at pH 6.2 were removed by this treatment. Reinecke salt was then extracted from the precipitate with acetone containing 1% thiodiethanol. The histone preparation was washed once more with acetone only and dried under reduced pressure.

Whole histone was separated by AUT-PAGE (15% T, 0.66% C). A narrow lane containing H3.1 and H3.2 was sliced out and mounted on top of an SDS-PAGE (15% T, 2.6% C) gel. The polypeptide fraction H3.2 was then recovered by electroelution as described elsewhere [14].

2.4. *Analytical two-dimensional electrophoresis of whole histone and sequence analysis of purified peptide fractions*

The procedures used have been described at length previously [8].

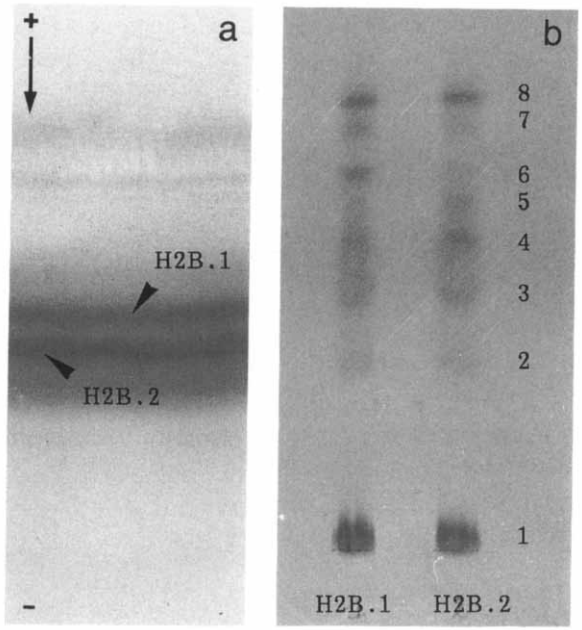

Fig.1. (a) Separation of the H2B isohistones of *C. elegans* by preparative AUT-PAGE (12% T, 0.66% C). (b) Analytical HPTLC of the peptides released after digestion of H2B.1 and H2B.2 with *Staphylococcus aureus* V8 proteinase. A minute (1 μl) sample was applied onto a 10 × 10 cm HPTLC-cellulose glass plate and developed in pyridine/butan-1-ol/acetic acid/water (10:15:3:12, by vol.). The position of the peptides was revealed with ninhydrin spray reagent. Peptides were numbered according to their increasing mobilities.

3. RESULTS AND DISCUSSION

3.1. *Identification of H2B subtypes*

When we originally sequenced the peptides released after digestion of unfractionated H2B with *Staphylococcus aureus* V8 proteinase and separated by preparative HPTLC on cellulose plates, we identified two peptides (Sa 5 and Sa 6) which differed from each other in only one single residue, i.e. alanine in Sa 6 and serine in Sa 5. In addition, two apparent heterogeneities

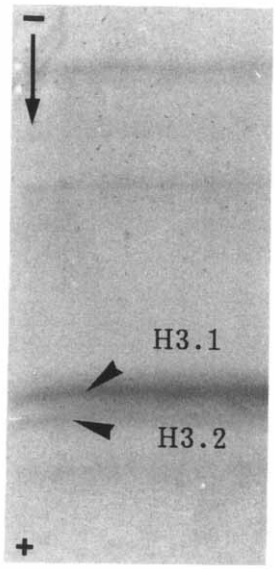

Fig.2. Separation of H3.1 and H3.2 polypeptides by preparative SDS-PAGE (15% T, 2.6% C).

were detected in the N-terminal Sa 1 peptide (Lys/Ala in positions 7 and 14) [6]. We have subjected both H2B isoforms to exactly the same treatments to obtain a rapid answer to the principal question of which residues belong to which polypeptide chain.

The *Staphylococcus aureus* V8 proteinase digest of the H2B fractions yielded one peptide fraction (Sa 6), which was predominant in the protein fraction designated H2B.1 and another one (Sa 5), which was clearly more abundant in H2B.2 (fig.1b). Automated sequence analysis of these peptide fractions provided the following sequence data:

Sa 6 (H2B.1): R-I-A-A-E

Sa 5 (H2B.2): R-I-A-S-E

Automated Edman degradation of both Sa 1 (fig.1b) peptides, which represented the N-terminal peptide fragments of H2B, provided this sequence information:

```
                          7                  14
Sa 1 (H2B.1): A-P-P-K-P-S-A(+K)-K-G-A-K-K-A-K(+A)-K-...

                          7                  14
Sa 1 (H2B.2): A-P-P-K-P-S-A(+K)-K-G-A-K-K-A-A(+K)-K-...
```

The finding that H2B.1 carries one more positive charge than H2B.2 is also supported by the slight but consistently higher mobility of H2B.1 in AUC gel (see fig.4A,C,E). We interpret the increase of the PTH signal of the amino acids given within brackets at position 14 of the amino acid chain to reflect cross-contamination of the H2B.1 and H2B.2 preparations. This is also supported by the cross-contamination of the H2B isohistone-specific peptides Sa 5 and Sa 6 in the fractions H2B.1 and H2B.2 (fig.1b).

The heterogeneity observed at position 7 of the amino acid chain is less clear. It was obtained in a ratio of approximately 3:1 (710 vs 240 pmol) i.e., quite similar to the ratio of Ala/Lys obtained at residue 7 using unfractionated H2B [6]. We found again that 10–14% of the H2B molecules lacked the N-terminal alanine residue, so an increase of the PTH-Lys signal by 70–100 pmol should be expected, being actually derived from Lys_7 of the shorter chain. This leaves some 100–140 pmol unexplained. We have previously [6] interpreted such a result as suggestive of a real Ala/Lys heterogeneity at position 7 of the major polypeptide chain (position 6 of the minor chain). However, if a portion of both H2B.1 and H2B.2 polypeptides might contain one more positive charge, this should be visible as distinct spots, migrating slightly ahead of the major spots, in a way the single acetylated forms of H2B.1 and H2B.2 are clearly distinct spots travelling just behind the major spots because they have one less positive charge. No such pattern is visible on the gels shown in fig.4A,B,C. For that reason we now assume that position 7 of the major

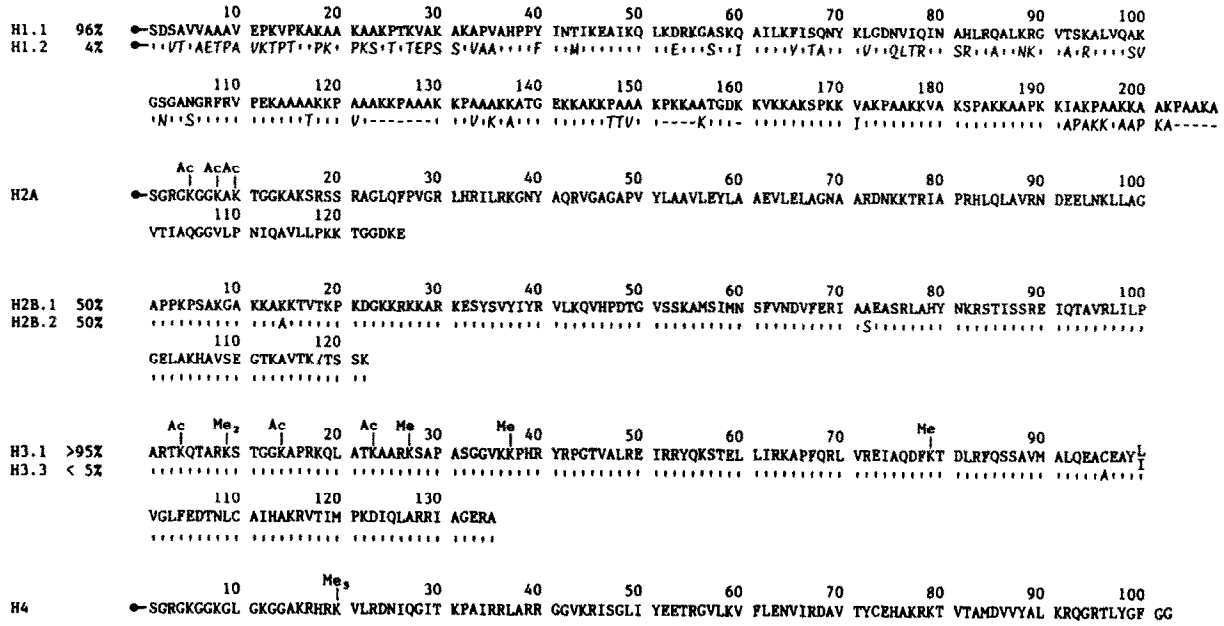

Fig.3. Relative abundance and amino acid sequences of histone isoforms expressed in *C. elegans*. Compilation of sequences taken from references: H2B [6], H4 [7], H3 [8], H2A [9], H1.1 [10] and H1.2 [14] and the additional data presented in this study. The relative amounts are derived from quantitative data when available e.g., relative yields by weight (H1 subtypes), relative yields of PTH amino acids at a polymorphic locus (H2B subtypes) or they are estimated from Coomassie staining intensities (H3). The figures listed have therefore an indicative value only. Sequence positions that differ from the major isoform are indicated in script, unaltered residues are indicated as ('), deletions as (–). (●–) N-terminal blocking group; Ac, acetylation; Me, Me_2, Me_3, 1–3 methyl groups, respectively. Modified residues were not studied in H2B. Note: in fig.1 of the original paper describing the primary structures of H4 [7], the glycine residue at position 28 was erroneously skipped.

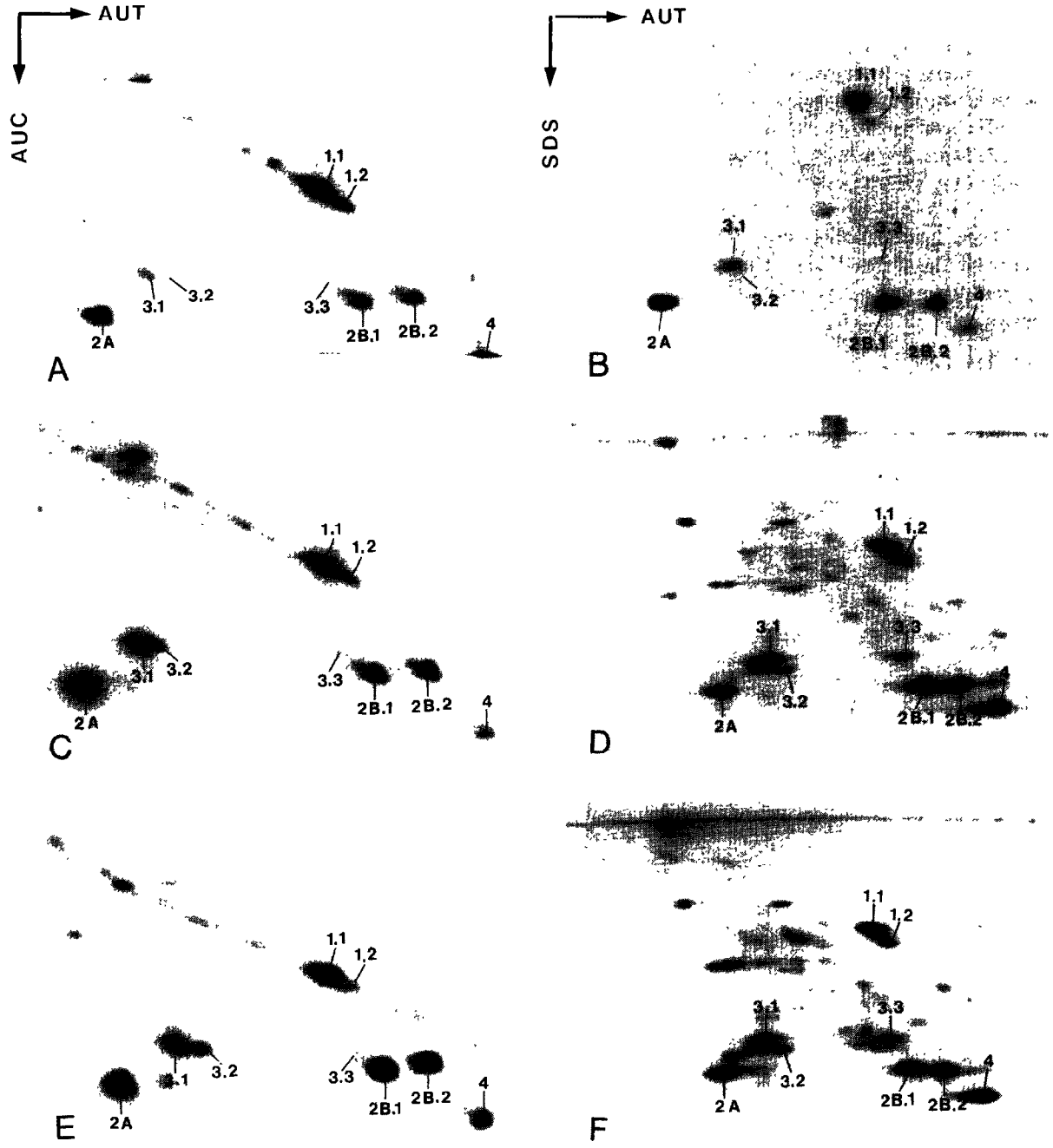

Fig.4. Two-dimensional separation of whole histone from age-synchronized *C. elegans*. The first dimension was AUT-PAGE (12% T, 0.66% C) and the second dimension was either AUC-PAGE (15% T, 0.66% C) or SDS-PAGE (15% T, 2.6% C). (A,B) Histones prepared from eggs; (C,D) histones from young adult (gravid) nematodes; (E,F) histones from senescent worms. The unlabeled spots are non-histone proteins that were co-extracted along with the histones.

chain (6 of the minor chain) in both H2B.1 and H2B.2 is only occupied by alanine and that there are only two isoforms of H2B (neglecting chain length polymorphism), i.e. H2B.1 with lysine in position 14 and alanine in position 72 of the major chain and H2B.2 with alanine and serine in the positions 14 and 72 of the major chain, respectively. Further work on the molecular cloning of the H2B genes may eventually reveal whether the chain length polymorphism is genuine or results from limited aminopeptidase activity.

3.2. *Characterization of H3.2*

Automated Edman degradation of fraction H3.2 (fig.2) provided the following sequence information: Ala-(?)-Thr-Lys-Gln-Thr-Ala-..., i.e. exactly the same sequence identified at the N-terminus of H3.1 [8]. Since the molecular mass of H3.2 is 700–900 Da smaller, it seems that H3.2 is a form of H3.1 which is shortened by proteolytic cleavage of approximately 6–8 amino acid residues at the C-terminus. It was surprising to see that cleavage has proceeded in spite of all precautions

taken to prevent in vitro proteolysis. Possibly H3.2 may result from processing in vivo, as suggested previously [8], and we cannot exclude the possibility that H3.2 might be encoded by a shorter gene. The molecular cloning of all H3 genes may provide a definite answer to this question.

3.3. *Expression of isoforms and degree of post-translational modification at different stages of the life cycle*

Fig.3 presents an overview of the histone variants that have been detected in *C. elegans*.

An important question concerns the possible role of the histone isoforms, e.g. does their expression coincide with distinct developmental or terminal differentiation stages? We have compared the two-dimensional gel pattern of the histone proteins expressed in eggs (these contain all stages of embryonic development, from simple zygote up to the first juvenile stage, which consist of 558 cells at hatching), young gravid adults (containing terminally differentiated somatic tissue, proliferating gonadal cells and eggs as well) and postreproductive, senescent worms (containing terminally differentiated cells only). Clearly, the expression of histone variant proteins remains essentially unaltered in the course of the life cycle (fig.4). There is, however, a decline of the extent of histone acetylation as a function of terminal differentiation and aging. This effect was consistently observed in duplicate experiments. Acetylation at multiple lysine residues removes positive charges. Such modified forms are visible on AUT/AUC two-dimensional gels as satellite spots on a diagonal behind each parental spot. The degree of acetylation is high in histones prepared from eggs (the full extent of acetylation is not visible on the photograph depicted in fig.4A because less protein was loaded and the very faint spots were lost photographically) and gravid adults. Histones prepared from postreproductive worms are poorly acetylated. In particular, H2A and both H2B isoforms seem to be almost devoid of acetylation in old worms.

Acetylated forms of histone are generally enriched in transcriptionally active chromatin [15]. On the other hand, nuclear activity generally declines with aging, so the decrease of histone acetylation in old worms seems straightforward. Alternatively, the decrease of histone acetylation with age may be simply due to reduced acetylase activity.

Acknowledgements: This work was supported by Grant 3.0071.84 from the Fund for Medical Scientific Research awarded to J.V. and J.V.B., and Grant 2.0042.85 from the Fund for Joint Basic Research awarded to J.V.B. J.V. acknowledges a Research Director career award with the National Fund for Scientific Research. This paper is a contribution to the Belgian National Incentive Program on Fundamental Research in Life Sciences initiated by the Belgian Science Programming Department.

REFERENCES

[1] Rykowski, M.C., Wallis, J.W., Choe, J. and Grunstein, M. (1981) Cell 25, 477–487.
[2] Grunstein, M., Rykowski, M., Kolodrubetz, D., Choe, J. and Wallis, J. (1984) in: Histone Genes. Structure, Organization & Regulation (Stein, G.S. et al. eds) pp.35–63, John Wiley and Sons, Chichester.
[3] Simpson, R.T. (1981) Proc. Natl. Acad. Sci. USA 78, 6803–6807.
[4] Wu, R.S., Tsai, S. and Bonner, W.M. (1982) Cell 31, 367–374.
[5] Meheus, L.A., Van Beeumen, J.J., Coomans, A.V. and Vanfleteren, J.R. (1987) Biochem. J. 245, 257–261.
[6] Vanfleteren, J.R., Van Bun, S.M., Delcambe, L.L. and Van Beeumen, J.J. (1986) Biochem. J. 235, 769–773.
[7] Vanfleteren, J.R., Van Bun, S.M. and Van Beeumen, J.J. (1987) Comp. Biochem. Physiol. 87B, 847–849.
[8] Vanfleteren, J.R., Van Bun, S.M. and Van Beeumen, J.J. (1987) FEBS Lett. 211, 59–63.
[9] Vanfleteren, J.R., Van Bun, S.M. and Van Beeumen, J.J. (1987) Biochem. J. 243, 297–300.
[10] Vanfleteren, J.R., Van Bun, S.M. and Van Beeumen, J.J. (1988) Biochem. J. 255, 647–652.
[11] Vanfleteren, J.R. and Meheus, L.A. (1988) Comp. Biochem. Physiol. 91B, 103–110.
[12] Johnson, K. and Hirsch, D. (1979) Dev. Biol. 70, 241–248.
[13] Lindh, N.O. and Brantmark, B.L. (1965) Anal. Biochem. 10, 415–420.
[14] Vanfleteren, J.R., Van Bun, S.M., De Baere, I. and Van Beeumen, J.J. (1989) Biochem. J., in press.
[15] Weisbrod, S. (1982) Nature 297, 289–295.