A novel linker histone-like protein is associated with cytoplasmic filaments in *Caenorhabditis elegans*

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Accepted 1 May 2002
Journal of Cell Science 115, 2881-2891 (2002) © The Company of Biologists Ltd

Summary

The histone H1 complement of *Caenorhabditis elegans* contains a single unusual protein, H1.X. Although H1.X possesses the globular domain and the canonical three-domain structure of linker histones, the amino acid composition of H1.X is distinctly different from conventional linker histones in both terminal domains. We have characterized H1.X in *C. elegans* by antibody labeling, green fluorescent protein fusion protein expression and RNA interference. Unlike normal linker histones, H1.X is a cytoplasmic as well as a nuclear protein and is not associated with chromosomes. H1.X is most prominently expressed in the marginal cells of the pharynx and is associated with a peculiar cytoplasmic cytoskeletal structure therein, the tonofilaments. Additionally H1.X::GFP is expressed in the cytoplasm of body and vulva muscle cells, neurons, excretory cells and in the nucleoli of embryonic blastomeres and adult gut cells. RNA interference with H1.X results in uncoordinated and egg laying defective animals, as well as in a longitudinally enlarged pharynx. These phenotypes indicate a cytoplasmic role of H1.X in muscle growth and muscle function.

Key words: *Caenorhabditis elegans*, Histone H1, Chromatin, Intermediate filaments, Linker histone

Introduction

Linker histones are highly abundant eukaryotic chromatin proteins. They bind to the nucleosomes, forming the 30 nm chromatin fiber. The biological function of linker histones is not sufficiently clear. In contrast to the core histones, which are essential for eukaryotic life (Han et al., 1987; Kim et al., 1988), linker histones are dispensable in single celled organisms like *Tetrahymena* (Shen et al., 1995) and *Saccharomyces cerevisiae* (Escher and Schaffner, 1997) as well as in simple multicellular fungi like *Aspergillus* (Ramon et al., 2000) and *Ascobolus* (Barra et al., 2000). In higher multicellular eukaryotes, linker histones usually exist as a set of relatively divergent protein variants. Although divergent, the structures of these protein variants follow certain canonical rules. They possess a short N-terminal domain and a longer C-terminal domain that is rich in lysine, alanine and proline residues. These structures are highly charged and bind by electrostatic interaction in a non-sequence-specific manner to linker DNA. The exact sequence of these terminal domains is not highly conserved, but a statistical analysis revealed that an even distribution of the charged lysine residues is a main principle (Subirana, 1990). The central conserved domain of the linker histone folds into a globular structure, the winged helix (Clark et al., 1993), which binds to the nucleosome. The occurrence of a typical linker histone gene family is correlated with multicellularity in plants as well as in animals. It is therefore conceivable that individual linker histone variants specifically contribute to essential aspects of higher multicellular life, such as cell differentiation and development.

We used *C. elegans* as a model system to investigate this question. *C. elegans* possesses eight different linker histone variants, the same number as human (Albig et al., 1997; Yamamoto and Horikoshi, 1996) or mouse (Drabent et al., 1995), and it allows us to dissect the function of individual linker histone genes with RNA interference (RNAi) and cytological techniques. In previous work we characterized the function of the major histone H1 variant in *C. elegans*, H1.1 (Jedrusik and Schulze, 2001). Depletion of H1.1 leads to a loss of the germ-line-specific chromatin silencing and consequently also to hermaphrodite sterility. Although the depletion of H1.1 was created with relatively low penetrance (11%) we believe that H1.1 is essential for the reproductive growth of *C. elegans.* Interestingly, however, the biological function of H1.1 is not a general housekeeping activity, such as maintaining the basic structures of chromatin, the nucleosome and the 30 nm fiber would suggest, but instead a specific contribution to a developmental program, the silencing and the maintenance of the germ line.

In this study we address the function of H1.X, a linker histone-like protein and a member of the *C. elegans* linker histone gene family. Although H1.X does follow the canonical three-domain structure of the tripartite linker histones, it violates further principles of linker histone structure. In contrast to all regular linker histones, H1.X does not contain lysine in its N-terminal domains. Additionally, and also in contrast to regular linker histones, H1.X contains the highly

hydrophobic amino acid residues tyrosine and leucine in both terminal domains. These drastic changes of the biochemical properties of its terminal domains suggest that histone H1.X has a function different from that of a typical linker histone. The work presented here confirms this assumption. RNA interference suggests that H1.X contributes to muscle function and development. H1.X is the first linker histone-like protein with a prominent cytoplasmic localization and function.

Materials and Methods

Strains

The general maintenance and routine culturing of *C. elegans* strains were performed as described previously (Brenner, 1974). The wild-type strain N2 (Bristol) was used for most experiments. *unc-65(e351)* was obtained from the *Caenorhabditis* Genetics Center (CGC). The *H1.X::GFP*-expressing transgenic reporter strains EC104, EC105 and EC106 were generated in this study. For this the histone H1.X-encoding gene was amplified by PCR from cosmid clone C30G7 (kindly provided by Alan Coulson, The Sanger Center, Cambridge, UK) with the primers MJ12 5′-CGGGATCCCGACAATAGCTC-TTTCTGGTTCCGG-3′ and ESMG34 5′-CGGGATCCCGTTCGTC-GGTCTCCCAGTC-3′. PCR was carried out using a proofreading enzyme [Expand High Fidelity PCR System (Boehringer)]. The product of 3.8 kb was inserted into pEGFP-N1 (Clontech) using the *Bam*HI restriction site of the polylinker. The resulting construct contains the *C. elegans* H1.X promotor and expresses the complete H1.X protein as a C-terminal fusion with GFP (green fluorescence protein). Extrachromosomal arrays were created by germ line transformation with the technique described by (Mello et al., 1991) using a blunt-ended mixture of linearized restriction fragments [50 μg/ml *Pvu*II-cut *C. elegans* genomic DNA, 0.5 μg/ml *Sca*I-cut *rol-6(su1006)* marker plasmid pRF4 (Kramer et al., 1990) and 1.9 μg/ml *Stu*I-cut *H1.X::gfp*].

cDNA cloning and RNA interference

A cDNA encoding H1.X (pC18/19) was cloned into pUC18 after reverse transcribed PCR amplification with the primers ESMG19 5′-GGATCCAGCATATGACCACTTCGCTCATCCACATGG-3′ and ESMG18 5′-CCATCGATGGTGGGAAATCTAAACTACAGGTGTC-3′ from total RNA of wild-type *C. elegans* using Superscript II polymerase (GibcoBRL) according to the manufacturer's instructions. For dsRNA synthesis the insert was transferred as a *Bam*HI-*Cla*I restriction fragment to pBluescript II SK (+) (Stratagene). Later, a second cDNA clone (yk480h7) encoding the same protein was kindly provided by Yuji Kohara, Mishima. The phenotype of H1.X depletion was generated with RNA-mediated interference (Fire et al., 1998). For the synthesis of double-stranded RNA (dsRNA), the plasmid template DNAs were separately digested with *Bam*HI and *Cla*I. The restriction enzymes were deactivated at 80°C for 20 minutes. The digested DNA templates were mixed together and transcribed in a single reaction using T7 and T3 RNA polymerases (Megascript T7 kit, Ambion). RNA integrity was determined by gel electrophoresis; concentrations were determined by ethidium bromide staining in the gel. Uncapped dsRNA H1.X with a concentration of 5 mg/ml was injected into hermaphrodite gonads of the wild-type strain. Unrelated dsRNA (4 mg/ml GFP dsRNA) or phosphate buffer M9 (Sulston and Hodgkin, 1988) were injected in control experiments. The F1 progeny derived from 0 to 48 hours after injection were scored for embryonic lethality, morphology and behavior. The injected animals were cultured on RNA feeding plates (Timmons and Fire, 1998). For RNAi feeding experiments, H1.X cDNA was cloned between the T7 promoters of the L4440 feeding vector and transformed into *Escherichia coli* HT115(DE3). Feeding plates were prepared from HT115 cells induced with 0.4 mM isopropyl-β-D-thiogalacto-pyranosid (IPTG) for 4 hours at 37°C.

Bacterial expression of truncated and full length recombinant H1.X proteins

Recombinant proteins were produced with the T7 polymerase expression system (Rosenberg et al., 1987) in *E. coli* BL21. For the expression of full-length H1.X the cDNA clone yk480h7 was amplified with the primers ESMG18 and ESMG19 (see above), cut with *Nde*I and *Cla*I and cloned into pET3a. For the expression of the truncated H1.X protein (C-terminal 101 amino acid residues), the corresponding region of the cDNA clone pC18/19 was amplified with the primers ESMG54 5′-GGAATTCCATATGTCAGAAGTTCGTC-AGAA-3′ and ESMG18 5′-CCATCGATGGTGGGAAATCTAAACT-ACAGGTGTC-3′, cut with *Nde*I and *Cla*I and cloned into pET3a. Protein expression and extraction were performed as described before (Wisniewski and Schulze, 1994).

Antibodies

Two different rabbit antisera were generated. For the first (αH1.X-11), a synthetic peptide corresponding to the C-terminal eleven amino acids of H1.X (Glu-Leu-Arg-Thr-Gly-Thr-Arg-Lys-Ser-Tyr-Cys) was synthesized and coupled to the carrier protein hemocyanin, which then was used as the antigen. This peptide was chosen because it is unrelated to any other *C. elegans* protein, including the remaining seven linker histones. The second antigen was recombinant truncated H1.X (the C-terminal 101 amino-acid residues). 500 mg of antigen were used for the immunization of each rabbit in a series of three injections. Antigen injections and resulting antiserum collections for both sera were performed by Charles River (Kissleg, Germany). The IgG fractions of the sera were obtained with a protein G column using the MAbTrap GII Kit (Pharmacia Biotech). αH1.X-11 and αH1.X-101 were affinity-purified on SulfoLink (Pierce) columns, onto which 1 mg of the corresponding HPLC purified antigen (Zorbax 300SB-C18 HPLC column, Hewlett Packard) had been coupled. The antibody directed against H1.1-H1.5 and H1.Q has been described previously (Jedrusik and Schulze, 2001). This antibody is not reactive with H1.X.

Western blot analysis

The lysates of *C. elegans* were prepared by boiling living worms in SDS sample buffer and subsequently separating the lysates on a 12% SDS-polyacrylamide gel. Western blot analysis was performed according to the method of (Towbin et al., 1979). After transfer onto a nitrocellulose membrane the blot was blocked for 1 hour at room temperature with 0.1% Tween-20 and 5% dry milk powder in TBS (150 mM NaCl, 10 mM KCl, 10 mM Tris-HCl pH 7.6) and then washed with TBS. The blot was incubated with 0.3 μg/ml αH1.X-11 or 14 ng/ml αH1.X-101 in TBS overnight at 4°C and washed with 0.1% Tween-20 in TBS at room temperature. The detection step was performed with the Phototope HRP Detection Kit (New England Biolabs) with a secondary anti-rabbit antibody diluted 1:5000. The blotting membrane was exposed to a Kodak blue Xomat Xb-1 film.

Immunofluorescence

General procedures were performed as described previously (Miller and Shakes, 1995). The immunolabeling of embryos was done with the freeze-cracking procedure (Strome and Wood, 1982). Embryos were fixed for 20 minutes in –20°C methanol and transferred to –20°C acetone for a further 10 minutes. The slides were air dried and incubated with 1.5% BSA in TBS before the first antibody was applied. Labeling of adult *C. elegans* was done according to Finney and Ruvkun (Finney and Ruvkun, 1990). Worms were suspended in

ice-cold RFB buffer (160 mM KCl, 40 mM NaCl, 20 mM Na_2EGTA, 10 mM spermidine-HCl, 30 mM PIPES, pH 7.4 and 50% methanol). Fixation was done with 1% formaldehyde in RFB in three freeze-thaw cycles. The sample was incubated for 40 minutes on ice with agitation and then washed two times with TTB (100 mM Tris HCl, pH 7.4, 1% Triton X-100, 1 mM EDTA). The worms were resuspended in TBS with 1% beta-mercaptoethanol, incubated for 2 hours on a shaker at 37°C and then washed in 10 volumes of borate buffer (1M H_3BO_3, 0.5 M NaOH, pH 9.5) with 0.01% Triton X-100. The worms were incubated for 15 minutes in borate buffer with 10 mM DTT and 0.01% Triton X-100 and washed again in 10 volumes of borate buffer with 0.01% Triton X-100. The samples were then incubated in borate buffer with 0.3% H_2O_2 and 0.01% Triton X-100 for 15 minutes at room temperature and once again washed with 0.01% Triton X-100 in borate buffer. After this step, the worms were washed with AbB (1× PBS, 0.1% BSA, 0.5% Triton X-100, 0.05% sodium azide and 1mM EDTA) for 15 minutes. The affinity-purified polyclonal αH1.X antibodies were used with a concentration of 6 μg/ml (αH1.X-11) or with 10.75 μg/ml (αH1.X-101) in AbA (AbB with 1% BSA), 10% goat serum and 0.1% Triton X-100. The samples were incubated overnight at 4°C. Fibrillarin was colocalized with ascites fluid of the mouse monoclonal antibody P2G3 (Christensen and Banker, 1992), diluted 1:3000. The samples were washed for 2 hours with several changes of AbB. The green fluorescent secondary antibody [Cy2-conjugated goat anti-rabbit IgG $F(ab')_2$ fragment, Jackson ImmunoResearch Laboratories] was diluted 1:800 with AbA; the red fluorescent antibody anti-mouse IgG-Cy3 conjugate (Sigma, C-2181) was diluted 1:100 with AbA and both were incubated overnight at 4°C.

As an accessibility control, a mouse monoclonal antibody [IFA (Bartnik et al., 1986)] directed against intermediate filament proteins was applied in combination with the αH1.X-11 antibody as a 1:1 diluted cell culture supernatant. IFA was detected with anti-mouse IgG-Cy3 conjugate (Sigma, C-2181) diluted 1:100. The specimens were stained either after the antibody incubations or directly after fixation with 1.6 μM 2′-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1 H-benzimidazole (Hoechst No. 33342) in PBS to visualize DNA.

Microscopy

Conventional and confocal light microscopy were performed with a Zeiss Axioplan 2 microscope equipped with a Zeiss confocal laser-scanning module LSM 510, Zeiss laser scanning software LSM 510 Release 2.01, a Spot RT CCD camera (Diagnostic Instruments, Sterling Heights, MI), Nomarski differential interference contrast and epifluorescence optics. Green fluorescent images were acquired with an excitation wavelength of 488 nm and an emission filter band pass 505-550 nm; Hoechst and DAPI DNA staining images were acquired with an excitation wavelength of 365 nm and an emission filter band pass 395 nm. Fig. 2A-E, Fig. 4, Fig. 5 and Fig. 6 are laser-scanning micrographs, whereas Fig. 2F, Fig. 3, Fig. 7, Fig. 8 and Fig. 9 were recorded using a conventional microscopic setup. Fig. 2A is a projection calculated from a stack of 71 individual two dimensional laser scannings recorded with a resolution of 1024×1024, which corresponded to 0.13 μm per pixel.

H1.X::GFP expression in HeLa cells

For the expression of H1.X::GFP in human HeLa cells (ATCC CCL-2), the H1.X cDNA was amplified with the primers ESMG73 5′-GGGGTACCACCATGGTGACCACTTCGCTCATCCACATGG-3′ and MJ12 5′-CGGGATCCCGACAATAGCTCTTTCTGGTTCCGG-3′ from the cDNA clone yk480h7 and cloned with *Kpn*I and *Bam*HI into the vector pEGFP-N1 (Clontech). The resulting plasmid expresses H1.X as an unfused protein from the CMV promotor and was used for the transfection of HeLa cells with lipofectamine (GibcoBRL) according to the manufacturer's instruction. The cells were seeded onto coverslips and exposed for 24 hours to the DNA-loaded liposomes in Optimem medium and cultured a further one or two days in DMEM with 10% FCS before microscopy was performed. Microscopy of living cells was done in PBS. Alternatively the cells were washed two times in PBS, fixed for 5 minutes with 4% formaldehyde in PBS (pH 7.6) at 25°C, washed with PBS and extracted with 1% Triton TX-100 in PBS for 20 seconds.

Computer software

Database searches in GenBank were done with the BLAST program suite version 2.2.1; database searches in the *C. elegans* databases were done with BLAST version 2.0a13MP (Altschul et al., 1990). The profile hidden Markow model database Pfam 6.5 and the software HMMER 2.1.1 (Bateman et al., 2000) were used for the protein domain analysis. Protein fold prediction was done with 3D-PSSM (Kelley et al., 2000). A final procession of micrographs was performed with the spot camera software 3.1 and Adobe PhotoShop 5.5.

Results

H1.X shares most structural characteristics of linker histone proteins but features also distinct and peculiar differences

The linker histone complement of *C. elegans* consists of eight proteins, H1.1-H1.6, H1.Q and H1.X as described by Jedrusik and Schulze and displayed in an alignment (Jedrusik and Schulze, 2001). The first six proteins represent typical linker histones with the canonical three-domain structure. Histone H1.Q is a small peptide of 60 amino-acid residues with high homology to the C-terminal domains of all remaining *C. elegans* histone H1 proteins. Histone H1.X shares the canonical three domain structure; its terminal domains, however, are highly divergent in their primary structure as well as in their amino-acid composition. We designated the name H1.X to this protein, because it is an extremely different linker histone. The primary protein structure of H1.X is defined by two cDNAs (pC18/19 and yk480h7), which both encode the same 25 kDa protein in accordance with the predicted protein C30G7.1 of the genome sequencing consortium. H1.X contains the complete structure of the central domain and all of the characteristic conserved amino-acid residues therein. Profile hidden Markow model analysis detects the 'linker histone' domain in H1.X with an expectation (E) value of 1.8×10^{-29}. The 'linker histone domains' in H1.1-H1.6 are detected with E values ranging from 3.7×10^{-31} to 3.4×10^{-42}. The 'linker histone domain' in the most divergent human linker histone, human H1.X (Yamamoto and Horikoshi, 1996), a protein unrelated to *C. elegans* H1.X, is detected with an E value of 1.7×10^{-10}, which shows that H1.X contains a structurally normal globular domain with a degree of divergence typical for linker histone gene families. This is independently confirmed by a protein fold recognition analysis with the complete H1.X sequence. The only fold detected by the 3D-PSSM algorithm (Kelley et al., 2000) is the linker histone winged helix domain (E-value: 6.5×10^{-6}).

The globular domain of H1.X is flanked by two terminal domains, whose size is comparable to that of the remaining linker histones of *C. elegans*. Consequently, H1.X is not the smallest nor the largest member of the linker histones gene family in *C. elegans*. The terminal domains of H1.X, however,

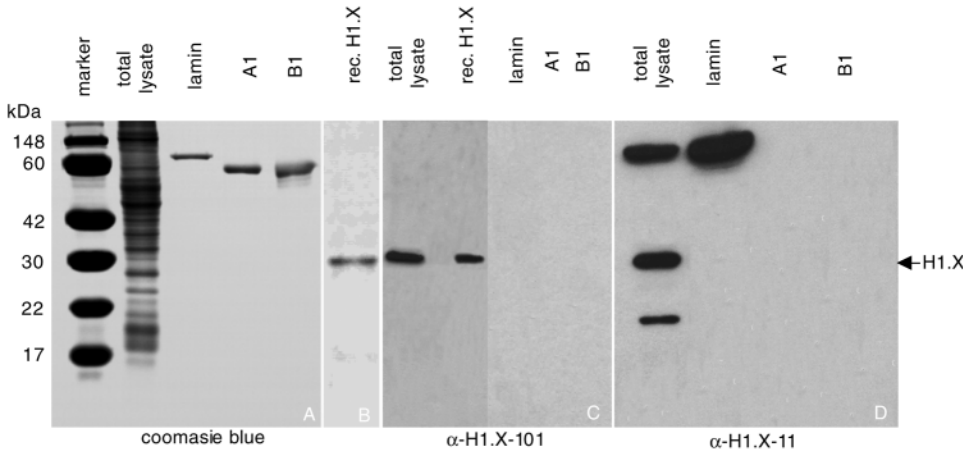

Fig. 1. Western blot analysis of histone H1.X. (A,B) shows Coomassie-blue-stained SDS page gels obtained from a total lysate of *C. elegans*, the recombinant *C. elegans* proteins lamin, intermediate filament proteins A1 and B1 and recombinant H1.X. (C) shows a corresponding blot stained with the αH1.X-101 antibody and (D) shows a corresponding blot stained with the αH1.X-11 antibody. No antibody is reactive with intermediate filament proteins. Both antibodies recognize H1.X as a band with an apparent molecular mass of 31 kDa. αH1.X-11 is crossreactive with *C. elegans* lamin and a further not identified protein.

contain features that are distinctly different from normal linker histone proteins. Usually both terminal domains are highly charged and contain about 40% lysine residues as well as high amounts of alanine, valine, proline, some residues of all other charged or polar amino acids, but no leucine, cysteine, histidine and strictly no aromatic amino residues. In contrast to this, the N-terminal domain of H1.X does not contain any lysine, but possesses one tyrosine, five leucine and five histidine residues. A BLASTP search in GenBank revealed that the N-terminal domain of H1.X is not only dissimilar to any other linker histone but also that it is dissimilar to any protein represented in the database.

In contrast to the N-terminal domain, the C-terminal domain of H1.X is relatively rich in lysine (22% compared with 28%-39% in H1.1-H1.5). It contains seven leucine residues, one tyrosine residue, two cysteine residues and two histidine residues. A BLASTP search performed on the *C. elegans* database Wormpep reveals that this C-terminal domain is related to the C-terminal domains of the remaining linker histones. Although the *C. elegans* linker histone genes encoding H1.1–H1.6 contain a single intron located at a conserved position in the globular domain, the gene encoding H1.X possesses this conserved intron as well as three additional introns located in all three domains of the protein. Therefore the structure of the gene encoding H1.X is clearly related to the remaining linker histones genes of *C. elegans*, but it also contains distinct and unusual structural differences.

The histone H1.X-encoding gene produces a single protein in *C. elegans*

Two independent polyclonal antibodies were raised either against a synthetic peptide or against a truncated recombinant H1.X protein. The synthetic peptide (the C-terminal 11 amino-acid residues of H1.X) was designed to prevent crossreactivity of the resulting antibodies with linker histone proteins. It does not contain lysine and arginine and is dissimilar to all other predicted *C. elegans* proteins. The truncated recombinant H1.X protein was designed as a larger and therefore more potent antigen which, however, bears the possibility of creating H1 crossreactive antibodies. Fig. 1 shows a western blot analysis of a *C. elegans* lysate with these two antibodies. Both antibodies recognize a common band of 31 kDa. In SDS-PAGE linker histones typically appear at molecular masses above their physical molecular masses because the high positive net charge is not completely shielded by the ionic detergent. The apparent size of H1.X (31 kDa) corresponds to that of the *C. elegans* linker histones H1.1, H1.3 and H1.5 (Jedrusik and Schulze, 2001), which have physical molecular masses of 21-23 kDa. The *C. elegans* H1 proteins H1.4 and H1.2 have an apparent molecular mass different from that of H1.X and are not detected by αH1.X-101 in the western blot experiment. This indicates that αH1.X-101, which was raised against the C-terminal domain of H1.X, does not show a general crossreactivity with *C. elegans* linker histones. This is confirmed by the observation that αH1.X-101 does not stain interphase chromatin or condensed chromosomes, like a normal anti-H1 antibody does.

The second antibody, αH1.X-11, is also not crossreactive with *C. elegans* linker histones but shows crossreactivity with two non-histone proteins of *C. elegans*. One of these bands appears at 22 kDa and represents an unidentified protein, whereas the other band at (60 kDa) is identified as lamin by direct comparison with recombinant *C. elegans* lamin protein (Liu et al., 2000) in the western blot. This comparison was performed because αH1.X-11 stains the nuclear lamina of all *C. elegans* nuclei in immunocytological preparations. Both antibodies do not react with intermediate filament proteins A1 and B1. As the 31 kDa band is exclusively recognized by both antibodies, and as this band co-migrates with the recombinant H1.X protein, we conclude that this band represents H1.X in *C. elegans*.

H1.X is tightly associated with the tonofilaments in the marginal cells

The most prominent structures labeled with both antibodies as well as with H1.X::GFP in transgenic animals are the marginal cells and the tonofilaments therein. The signal appears in all larval stages and in adult *C. elegans*. Fig. 2A shows a projection calculated from a stack of 71 confocal images from a hermaphrodite stained with αH1.X-11; Fig. 2B shows one of these sections in differential interference contrast. The antibody labeling procedure includes a detergent extraction/reduction step. Using these conditions the only labeled structures in the marginal cells are the tonofilaments. No further nuclear or cytoplasmic signal appears besides the

labeling of all nuclear lamina caused by the anti-lamin crossreactivity of αH1.X-11. This result is confirmed by independent antibody labeling (Fig. 2C) with αH1.X-101. In these preparations no nuclear signal appeared in the nuclei of the marginal cells, although the cytoplasm of some head neurons was stained (data not shown). αH1.X-101 did not label the nuclear lamina. In *H1.X::gfp* transgenic animals (Fig. 2D,F), the cytoplasm of the marginal cells appeared brightly fluorescent, and the nuclei therein became visible as the brightest fluorescent structures of the cells. The GFP fluorescence was almost homogeneously distributed throughout the whole cytoplasm, but the tonofilaments therein became clearly visible as bright green dots when visualized in axial orientation (Fig. 2F). A double labeling experiment was performed with αH1.X-11 and the monoclonal antibody IFA, which stains the intermediate filament proteins of the tonofilaments (Bartnik et al., 1986). Both antibodies label identical structures - the tonofilaments in the marginal cells (Fig. 3C,D). The different pictures of the marginal cells created by H1.X antibody labeling and H1.X::GFP fluorescence suggest that a fraction of H1.X::GFP fills the cytoplasm as well as the nucleoplasm as a highly soluble protein, whereas a further fraction tightly binds to the tonofilaments, a cytoskeletal structure created by the intermediate filament system. This hypothesis was tested by subjecting the *H1.X::gfp* animals to the fixation and detergent extraction/reduction protocol used for antibody labeling. This experiment was done in combination with the IFA antibody labeling and resulted in a double labeling of the tonofilaments (data not shown) and no remaining further signals in the cytoplasm or nucleoplasm. This observation confirms that a major fraction of H1.X::GFP is a highly soluble protein in the cytoplasm as well as in the nucleoplasm, which can be readily extracted with 1% Triton X-100 and 1% β-mercaptoethanol in two hours at 37°C.

H1.X::GFP is a soluble cytoplasmic and nucleoplasmic protein in muscle cells, neurons and in excretory cells

The *H1.X::gfp* transgenic *C. elegans* lines revealed H1.X expression in further cell types. H1.X::GFP is expressed in body-

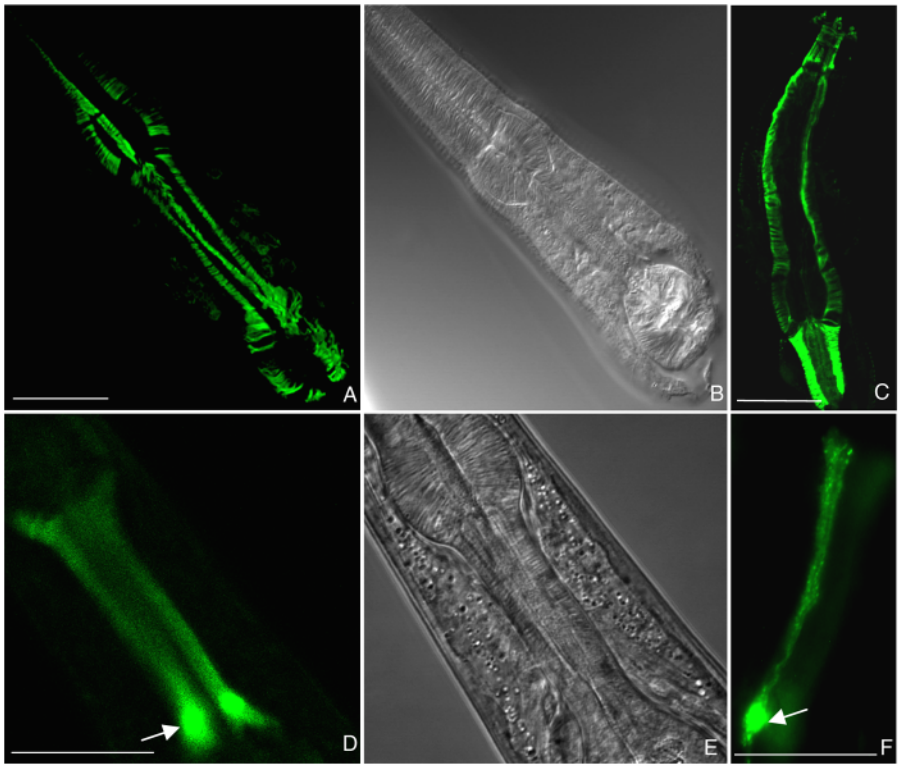

Fig. 2. H1.X detection in the marginal cells of the *C. elegans* pharynx. (A) represents an antibody labeling performed with αH1.X-11; (B) represents the corresponding Nomarski micrograph. (C) represents an antibody labeling performed with αH1.X-101. (D) shows H1.X::GFP expression in the marginal cells in a view lateral to the flat cell bodies. Here the tonofilaments are viewed in lateral orientation. (E) is the corresponding Nomarski micrograph. (F) shows H1.X::GFP expression in the marginal cells in an optical cross-section of the flat cell bodies. Here the tonofilaments appear in axial orientation as bright green fluorescent dots. H1.X::GFP shows a cytoplasmic as well as a nuclear localization (arrows point to the nuclei) of the fusion protein. Bar, 20 μm.

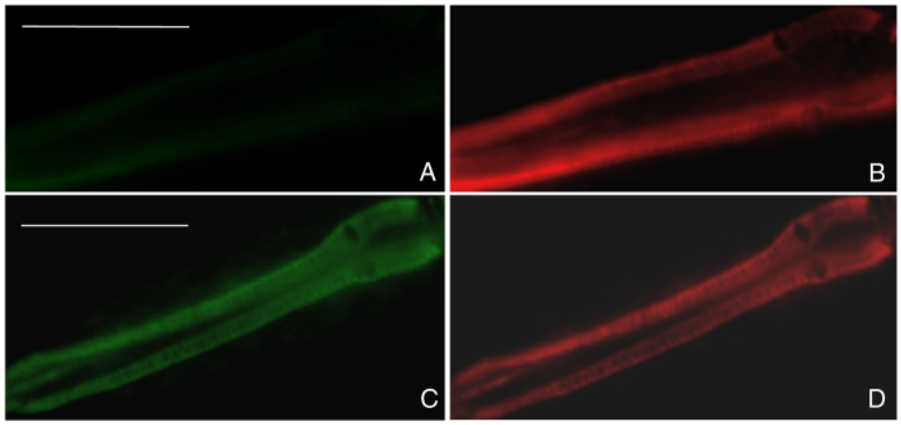

Fig. 3. H1.X depletion by RNA interference and colocalization of H1.X with the intermediate filament proteins in the marginal cells of the pharynx. Indirect immunofluorescence detection of histone H1.X in the marginal cells of the pharynx (αH1.X-11) shows a substantial reduction of H1.X after H1.X RNA interference (A) but not in control animals (C), which present intensely stained tonofilaments. The same two animals were additionally stained with the monoclonal anti-intermediate filament antibody IFA (B,D) (Bartnik et al., 1986), which decorates the tonofilaments. This shows a comparable permeabilization and antibody accessibility in both animals as well as a colocalization of both antigenes in the tonofilaments. Bar, 20 μm.

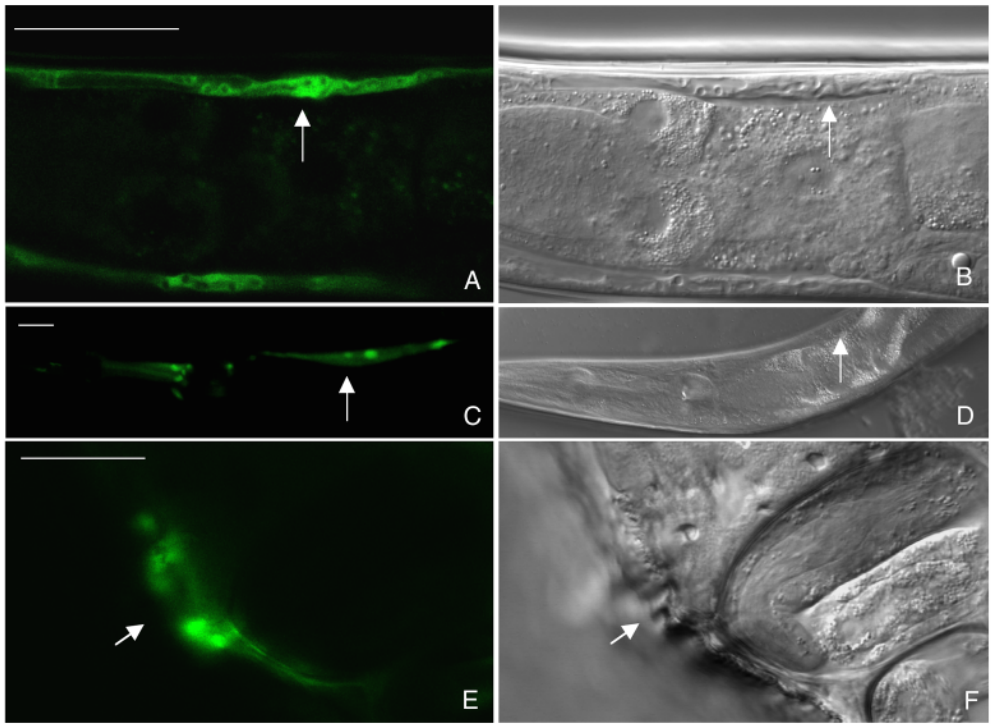

Fig. 4. The expression of histone H1.X::GFP in *C. elegans* body muscle cells (A,C) and in the vulva muscle cells (E). H1.X::GFP is prominently localized in the cytoplasm but also enriched in the nuclei. (B,D,F) represent the corresponding Nomarski micrographs. The arrows points to individual body-wall muscle cells and the nuclei therein and to the vulva opening. Bars, 20 μm.

wall muscles (Fig. 4A,C), as well as in the vulva sex muscles (Fig. 4E). In both cases the general appearance of the cells corresponds to the situation already described for the marginal cells of the pharynx: the fluorescence signal fills the cytoplasm, and the cells' nuclei appear as the brightest fluorescent structure. H1.X presence in muscle cells was not detected with the two different antibodies. We ascribe this to a complete extraction of H1.X from these cells during the extraction/reduction step of the antibody labeling procedure.

H1.X::GFP was also expressed in a limited number of head neurons (Fig. 5A), in which the fluorescence signal filled the total cytoplasm, including the neuronal projections. The presence of H1.X in these cells was confirmed with the αH1.X-101 antibody (data not shown). Furthermore, H1.X::GFP was detected in the cytoplasm of excretory cells (Fig. 5C).

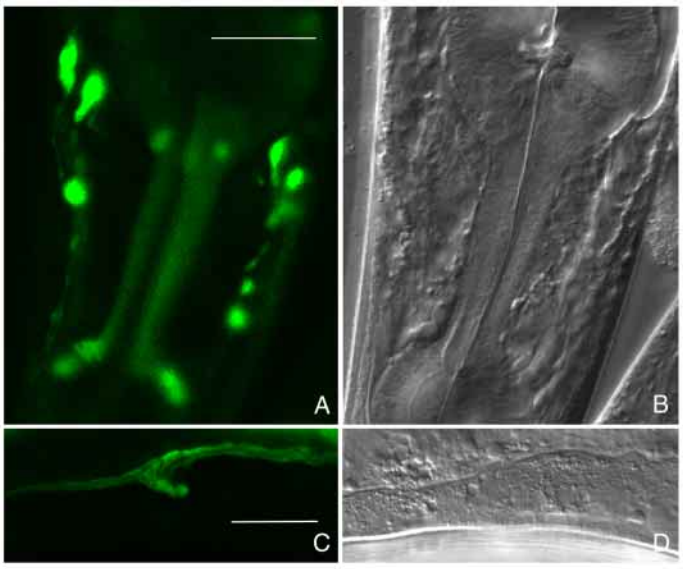

Fig. 5. The expression of histone H1.X::GFP in head neurons and marginal cells (A) as well as in an excretory channel cell (C). H1.X::GFP is a cytoplasmic protein in both cell types. The neuronal projections are visualized by H1.X::GFP fluorescence. (B,D) are the corresponding Nomarski micrographs. Bars, 20 μm.

H1.X is a nucleolar protein

In *C. elegans* embryos, H1.X::GFP expression starts with the 30-cell stage. Fig. 7A shows the expression of H1.X::GFP in four cells in the periphery of an embryo with more than 100 cells. This specimen is fixed. A shallow fluorescence signal fills the nucleoplasm, and the nucleoli appear brightly fluorescent. In Fig. 7B a comparable view of a living embryo is presented. Here the cytoplasm is filled with a shallow fluorescence signal, whereas the nuclei appear brightly fluorescent. The prominent green spots in the nuclei are the nucleoli, which were identified by differential interference contrast (DIC) microscopy (data not shown). These observations suggest that a fraction of H1.X::GFP is a highly soluble protein in the cytoplasm as well as in the nucleoplasm, whereas a different fraction binds tightly to the nucleolus. Antibody labeling with αH1.X-101 revealed a prominent labeling of the nucleoli in the polyploid gut nuclei (Fig. 6B) of adult *C. elegans* hermaphrodites, which colocalized with fibrillarin (Fig. 6D), a structural component of small nucleolar RNPs (snoRNPs) implicated in pre-rRNA processing. A prominent co-immunolabeling was further detected in the nucleoli of the germ nuclei and many other cell types and tissues in *C. elegans*. The nucleoli of the polyploid gut cells are comparatively deprived of DNA, shown by the

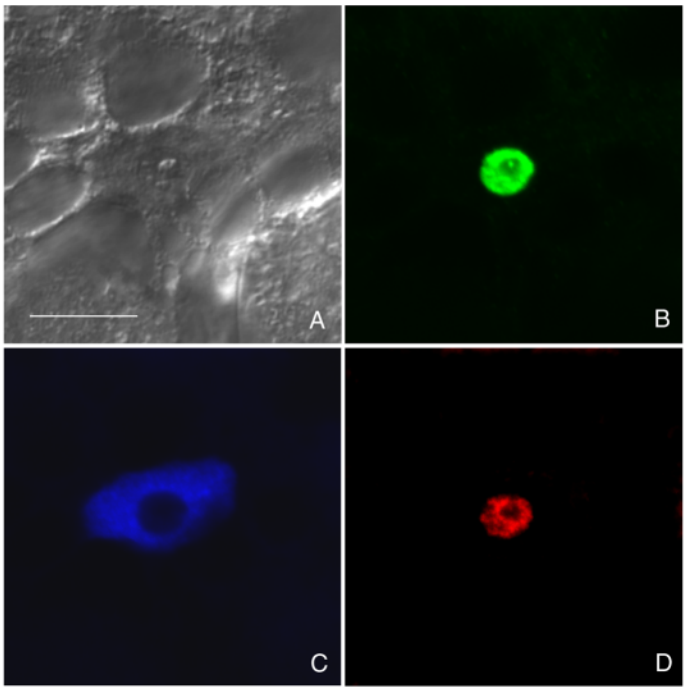

Fig. 6. Nucleolar localization of H1.X (A) shows a Nomarski micrograph of the nuclear region of a polyploid gut cell in a hermaphrodite *C. elegans* with the nucleolus and its substructures visualized by differntial interference contrast. (B) H1.X detected with the antibody αH1.X-101 localizes to the nucleolus; (C) corresponds to DAPI DNA staining; and (D) shows the indirect immunfluorescence detection of the nucleolar protein fibrillarin with the specific monoclonal antibody P2G3 (from M. Christensen). Bar, 10 μm.

dark regions in the DAPIstained nucleus (Fig. 6C). The nucleolar DNA (rDNA) was occasionally visible as faintly DAPI-stained ring-like structure in the confocal sections (data not shown). In all these cases the antibody staining of H1.X colocalized with fibrillarin, and it was not associated with the

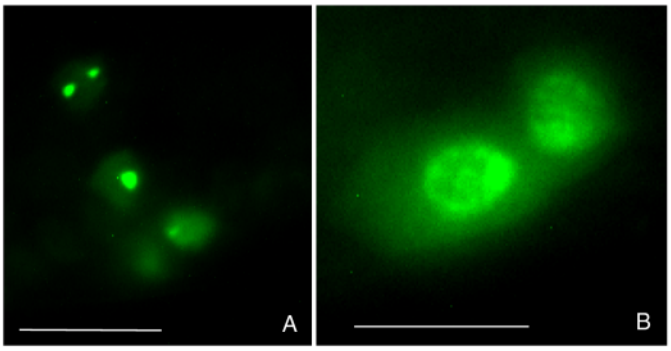

Fig. 7. Embryonic expression and subnuclear localization of H1.X. (A,B) show H1.X::GFP fluorescence detection in a few cells in the periphery of a >100-cell stage embryos. In (A), a fixed specimen shows a shallow fluorescence of the nucleoplasm and bright fluorescence of the nucleoli, whereas in (B), a live observation shows a shallow fluorescence in the cytoplasm and a bright fluorescence of the nucleoplasm. The brightest spots in the nucleoplasm correspond to the nucleoli. Bars, 20 μm.

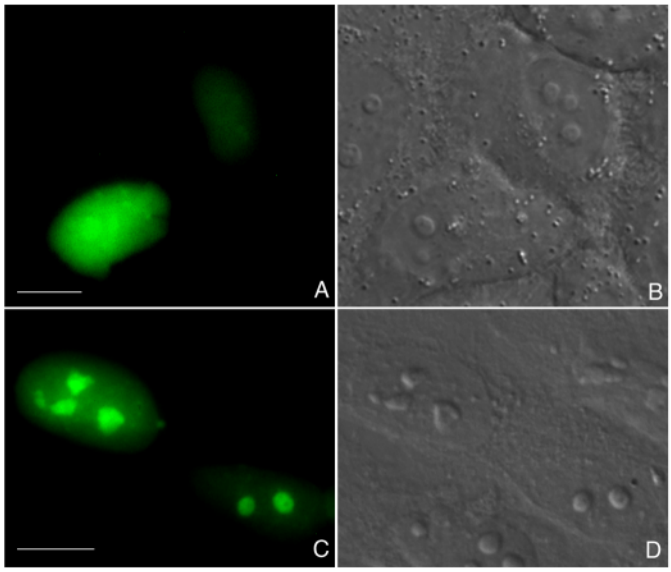

Fig. 8. Expression of H1.X::GFP in living (A) and in formaldehyde-fixed HeLa cells (C). The nucleoli are prominently fluorescent in the fixed and extracted cells, whereas in living cells the whole nucleoplasm fluoresces. The brightest spots in the nucleoplasm of the living cells correspond to the nucleoli. (B,D) represent the corresponding Nomarski micrographs. Bars, 10 μm.

nucleolar DNA. In contrast to the other H1 proteins of *C. elegans*, H1.X was never detected in condensed mitotic or meiotic chromosomes or as a structural component of the interphase chromatin, as revealed by antibody labeling (αH1.X-11) of embryos and meiotic oocytes. H1.X::GFP was never found localized to condensed chromosomes. This is in strict contrast to the properties of other H1 proteins in *C. elegans*. H1.1-GFP fluorescence is readily observed in condensed mitotic chromosomes in *C. elegans* embryos, and it is detected as a structural component of interphase chromatin in antibody labeling of embryonic blastomeres and all other cell types of *C. elegans*.

To investigate the relation of H1.X with cytoplasmic and nuclear substructures, H1.X::GFP was expressed in HeLa cells. Here H1.X::GFP does not appear in the cytoplasm but is a strictly nuclear protein (Fig. 8A). This observation indicates that the prominent cytoplasmic localization of H1.X::GFP in *C. elegans* is not caused by a general failure of the nuclear translocation of the fusion protein. When H1.X::GFP-expressing HeLa cells were fixed and Triton X-100 extracted, the GFP fluorescence of the nucleoplasm was strongly reduced and the nucleoli appeared brightly fluorescent (Fig. 8C). These results show that H1.X::GFP is a highly soluble protein in the nucleoplasm of HeLa cells and that it specifically binds to the nucleolus, like it does in *C. elegans*. In fixed as well as living HeLa cells H1.X::GFP is additionally localized in small spot-like structures, which may represent further nuclear bodies.

H1.X is involved in muscular function

RNA interference experiments were performed in order to analyze the function of H1.X in *C. elegans*. 1351 F1 animals obtained from 42 dsRNA microinjected mothers (F0) were

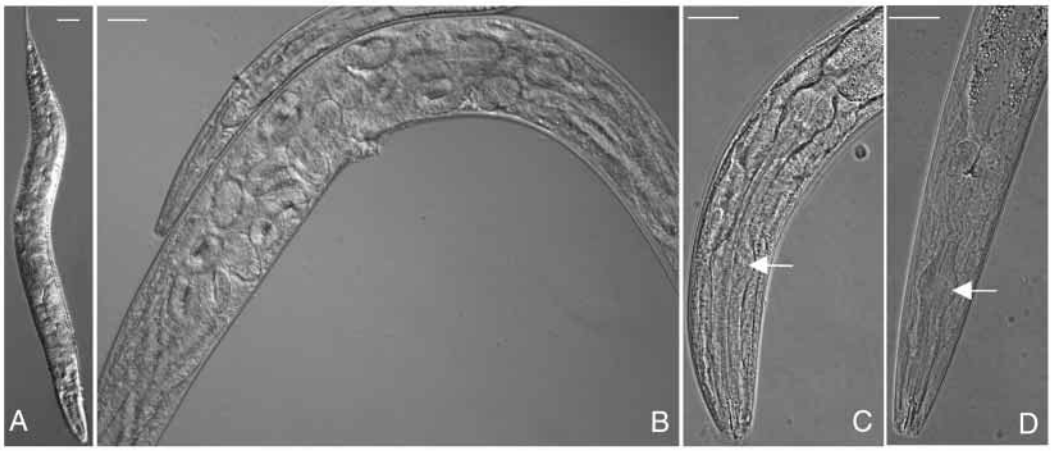

Fig. 9. *C. elegans* phenotypes created by H1.X RNA interference. 61 animals from 1351 scored F1 animals (4.5%) are small and dumpy (A). They reach only half of the final body length of normal *C. elegans*. Half of these animals (34 of 61) are additionally defective in egg laying (B). Another phenotype affected 3% of the F1 population (33 animals of 1351). These animals contain an abnormally elongated pharynx (C). A control animal is shown in (D; the arrows point to the pharynx). Bar, 20 μm.

scored. 4.5% of these (61 animals) showed a severe reduction of the final body size, a dumpy-like body appearance and a slow and uncoordinated mode of locomotion (Fig. 9A). 34 of these 61 animals were additionally defective in egg laying (Fig. 9B). The average final body length of these animals was 483±204 μm, whereas the average body length of the control group (five animals measured) was 925±55 μm. Additionally a further independent phenotype was observed. 33 animals (3% of the scored F1) possessed an elongated pharynx (Fig. 9C). This group is not overlapping with the small, dumpy-like and uncoordinated animals described first. The average pharynx length was 141.4±8.4 μm, whereas it was 125.4±5.5 μm in the control group (236 F1 animals obtained from five phosphate buffer injected mothers). All animals of the control group appeared to be wild-type according to their mode of movement, reached the normal body size and were never defective in egg laying. In order to assess the reduction of H1.X in the phenotypic animals, immunocytology was performed. The phenotype of the elongated pharynx was correlated with a severe reduction of the H1.X content of the marginal cells. Fig. 3A shows the reduction of H1.X in such an animal, whereas Fig. 3B presents a control labeling with the IFA antibody to demonstrate antibody accessibility. Fig. 3C,D present the corresponding labeling of a control animal. RNA interference reduced the H1.X content of the tonofilaments drastically, but a residual amount of the protein is clearly detected. 231 F1 adult hermaphrodites from a H1.X RNA interference experiment were analyzed with indirect immunofluorescence (αH1.X-11). Half of these animals showed a reduction of the intensity of the labeling of the tonofilaments to about 50% of the signal intensity of the control group. However, 10 animals (4.3% of the F1) showed a far stronger reduction of the H1.X signal obtained from the tonofilaments. This group corresponded to the animals that had an abnormally elongated pharynx. We conclude from these observations that H1.X contributes to the development of muscles and muscular organs in *C. elegans* as well as to basic muscular functions, like locomotion and egg laying. Alternatively some of these phenotypic properties could also be caused by a loss of H1.X in neurons. This seems to be not very probable, as RNAi-mediated depletion of proteins in neurons typically is not very effective.

The *C. elegans* mutant *unc-65(e351)* (Brenner, 1974) is slightly uncoordinated, slowly moving and occasionally egg laying defective. Because *unc-65(e351)* was mapped in very close vicinity to H1.X, we tested whether *unc-65(e351)* is H1.X. This was done by PCR amplification of the complete coding region of H1.X and subsequent DNA sequencing. This revealed a wild-type H1.X sequence in *unc-65(e351)*. Western blot analysis and immunocytology of H1.X in *unc-65(e351)* also produced data identical to the wildtype. We therefore conclude that *unc-65* is not H1.X.

Discussion

Linker histones (H1 and H5 proteins) are normally strictly nuclear proteins that never reside in the cytosol at detectable concentrations. Linker histones are associated with interphase chromatin as well as with condensed chromosomes. They are produced at S-phase in a cell-cycle-dependent manner and are co-replicationally incorporated into the chromatin. Additionally a further linker histone, the replacement type linker histone, can be produced constitutively (for a review, see Doenecke et al., 1994). The canonical three-domain structure of the linker histone is found in multicellular animals and fungi, in plants (including algae), but not in yeast and protozoa such as *Tetrahymena*. The occurrence of a typical linker histone multigene family is correlated with multicellularity in plants as well as in animals. Although characterized as a constitutive component of the eukaryotic chromatin, linker histones exchange at their binding sites at surprisingly high rates (Misteli et al., 2000; Lever et al., 2000). Knockouts performed in *Tetrahymena* (Shen et al., 1995) and fungi [*Saccharomyces cerevisiae* (Escher and Schaffner, 1997), *Aspergillus* (Ramon et al., 2000) and *Ascobolus* (Barra et al., 2000)] show that linker histones are dispensable for these organisms. For the fertility of the nematode *C. elegans*, however, a single linker histone variant seems to be essential (Jedrusik and Schulze, 2001). Similar observations have been made in plants (Prymakowska-Bosak et al., 1996; Prymakowska-Bosak et al., 1999). These results demonstrate that linker histones do not have general functions such as participation in the basic structural units of the chromatin, the chromatosome and the 30 nm fiber, but instead can contribute to specific programs. To elucidate the nature of these programs we used *C. elegans* as a model system.

In contrast to the linker histones and linker histone related proteins characterized so far, the linker histone-like protein H1.X resembles a tripartite linker histone that does not contain lysine in its N-terminal domain. By contrast, it contains multiple leucine, tyrosine, histidine and cysteine residues in its terminal domains. The DNA-binding forces of linker histones are generated by non-sequence specific electrostatic interactions between the numerous charged lysine residues of the linker histones with the phosphate di-ester groups of the DNA. Both terminal domains of normal linker histones are very rich in lysine residues and usually do not contain any highly hydrophobic amino acids, such as tyrosine, leucine, cysteine or histidine. These amino acids are prone to different types of side chain interactions, such as hydophobic interactions and the formation of covalent disulfide bonds. These types of interaction are not possible in normal linker histones and cannot directly serve the purpose of DNA binding. This suggests that the terminal domains of H1.X could be differently folded than the terminal domains of normal linker histones and that they could provide different types of chemical interactions, for example protein-protein interactions instead of DNA binding. Our cytological observations support this interpretation. H1.X is a prominent cytoplasmic protein in a limited number of cell types; these are the marginal cells, a set of nine epithelial cells in the pharynx (three of which form a syncytium), the body-wall muscle cells and the vulva muscles in the hermaphrodite. Additionally a limited number of head neurons and excretory cells also express H1.X.

The cytoplasm of the marginal cells is filled with thick bundles of intermediate filaments, the tonofilaments. H1.X is tightly associated with these tonofilaments. The presence of H1.X at the tonofilaments in the marginal cells was shown by indirect immunofluorescence with two independently raised polyclonal antibodies, H1.X::GFP fusion protein expression and a specific reduction of the immunofluorescence signal by H1.X RNA interference. Prominent and uniform cytoplasmic expression of H1.X was detected by indirect immunofluorescence in some head neurons. This observation was confirmed by a comparable expression of H1.X::GFP in these neurons. No visible filamentous structures were detected in the cytoplasm of these cells by confocal light microscopy. H1.X::GFP expression labeled additional cell types and produced a uniform fluorescence in the cytoplasm and in the nucleoplasm. Most of the H1.X::GFP fluorescence was removed by the extraction/reduction procedure necessary for antibody staining. This observation may explain why it was difficult to detect cytoplasmic H1.X expression in these cells using antibodies. Currently it can not be decided whether the differences in the distribution of antibody staining and H1.X::GFP fluorescence, as seen in the marginal cells, result from overexpression of H1.X::GFP (and therefore are artificial) or result from an extraction of native H1.X protein during the antibody staining procedure. Normal H1 proteins are strictly nuclear, and they are not removed from interphase chromatin or condensed chromosomes by the described extraction/reduction procedure.

Two different phenotypes were observed in the H1.X RNA interference experiments. In the first phenotype, which correlated with an immunocytologically detected depletion of H1.X in the pharynx, a longitudinally elongation of this muscular organ was observed. Animals that showed the second phenotype were small, slowly moving in an uncoordinated fashion and defective in egg laying. We believe that the latter phenotype results from a depletion of H1.X in sex and body-wall muscles. Interestingly, RNAi with the intermediate filament protein C2 also results in a dumpy phenotype with a slightly reduced motion at a penetrance of 10% (Karabinos et al., 2001). The expression pattern of intermediate filament protein C2 as well as some further members of this gene family are as yet not known. It is therefore conceivable that one of these proteins is expressed in body-wall and/or sex muscle cells. In the *Ascaris lumbricoides* intermediate filament, proteins are present in body-wall, pharyngeal and uterine muscle cells (Bartnik et al., 1986). These represent three differently striated muscle cell types. H1.X expression was furthermore detected in excretory cells and in some head neurons. In *C. elegans*, the intermediate filament proteins B1 and A4 are expressed in the excretory cells, and RNAi with the intermediate filament proteins A2 and A3 results in uneven excretory canals, body muscle displacement and paralysis (Karabinos et al., 2001). A neuronal expression has been reported for the intermediate filament protein A1 (Karabinos et al., 2001). It is therefore conceivable that the adult expression of H1.X is always associated with co-expression of intermediate filament proteins. H1.X, however, was never detected in the hypodermis, a tissue in which the intermediate filament system is of essential importance (Karabinos et al., 2001). We therefore propose that H1.X could be a facultative component of the intermediate filament system in *C. elegans*.

Linker histones can be seen as a facultative and non-essential assembly factor of the 30 nm chromatin fiber. The location of H1 in the filament, its role in filament formation and the structure of the 30 nm filament itself have all been controversial (for a review, see Ramakrishnan, 1997). A cytoplasmic- and filament-associated function of histone H1 has been suggested before. A somatic chromatin type histone H1 was shown to be associated with the sea urchin sperm flagellum, and histone H1 was recognized as a component of the cilia and their basal bodies in the ciliate *Paramecium* and in the green algae *Chlamydomonas reinhardtii* (Multigner et al., 1992). Unfortunately no further investigations have followed this initial report.

A recent theoretical analysis performed on all available linker histone gene structures demonstrated a correlation of the presence of flagellated gametes with the presence of tripartite non-polyadenylated linker histones in plants as well as in animals (Kaczanowski and Jerzmanowski, 2001). It is speculated by these authors that an evolutionarily early function of H1 might have been that of a mediator between specialized microtubules and chromosomes during mitosis. Interestingly we noted a significantly increased occurrence of males during the first generations of the H1.X::GFP expressing transgenic animals. Male genotypes (X0) originate from hermaphrodite mothers (XX) by non-disjunction of the X-chromosome. Germline expression of repetitive transgenes is typically lost after a few generations. This could indicate that H1.X::GFP expression interferes with normal chromosome segregation during meiosis.

Histone H1.X is a divergent member of the linker histone gene family of *C. elegans*. Current sequence data suggest that it is derived from normal linker histones in the line of nematodes and that it has no counterpart in other phyla. This

protein, however, demonstrates that relatively few changes are required to convert a linker histone into a cytoplasmic fiber-associated protein. Interestingly, cdc2 kinase, which is also known as histone H1 kinase (Arion et al., 1988), extensively regulates the structure of the cytoplasmic as well as of the nuclear intermediate filament system by phosphorylating vimentin (Tsujimura et al., 1994) and lamin (Heald and McKeon, 1990) in a cell-cycle-dependent manner. In addition cdc2 kinase also phosphorylates caldesmon (Yamashiro et al., 1991), a molecule that binds to tubulin (Ishikawa et al., 1992) and is an established regulator of the actin filament system (Sobue et al., 1981). Whereas the phosphorylation of the intermediate filament proteins by cdc2 kinase results in disassembly of the filaments, the role of H1 phosphorylation during mitosis is not understood. The coregulation of cytoplasmic filaments and chromatin by cdc2 kinase supports the idea of a present or past functional relation between the cytoskeleton and linker histones or linker histone-related proteins.

In many cells of *C. elegans* H1.X is associated with the nucleolus, where it co-localizes with fibrillarin, but not with rDNA. A linker histone variant, which is exclusively associated with the nucleolus, and which strongly binds to rDNA in vitro, has been described in a number of plants (Tanaka et al., 1999). Recently an unexpected extracellular function of mammalian histone H1 in muscle regeneration and proliferation has been described (Henriquez et. al., 2002).

Our present data suggest that H1.X is the first example of a protein resembling the general structure of a tripartite linker histone that probably is not a chromatin component and that interacts with other nuclear and cytoplasmic structures instead. We therefore think that the unusual facts presented here will promote the analysis of linker histone function and linker histone evolution in a general way.

We thank Ulrich Grossbach, Günter Schmahl and Dietbert Rudolph, Göttingen, for their support of the *C. elegans* group, Klaus Weber, Göttingen, for critical discussion of this work as well as for the IFA antibody and three recombinant *C. elegans* proteins, Alan Coulson, Hinxton, for the cosmid C30G7, Yuji Kohara, Mishima, for the cDNA clone yk480h7, Jonathan Hodgkin, Oxford, for *unc-65* alleles, Agniezka Patkaniowska and Stephan Fröde for sequencing H1.X of *unc-65(e351)*, M. Christensen for ascites fluid of mAB P2G3, Bettina Schulze for critically reading this manuscript and the students of our summer 2001 advanced level practical course in developmental biology for the production of full-length recombinant H1.X protein. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). We want to acknowledge the excellent technical assistance of Sabine Pitzel and Barbara Lage. This work was supported by the Deutsche Forschungsgemeinschaft grant GRK 242-2 (Graduiertenkolleg 'Molekulare Genetik der Entwicklung') to U. Grossbach and grant SCHU 1033/3-2 to E.S. and U. Grossbach, SCHU 1033/3-3 and SFB271 A14 to E.S.

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