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Most genes encoding cytoplasmic intermediate filament (IF) proteins of the nematode *Caenorhabditis elegans* are required in late embryogenesis

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Intestinal cells of *C. elegans* show an unexpectedly high complexity of cytoplasmic intermediate filament (IF) proteins. Of the 11 known IF genes six are coexpressed in the intestine, i.e. genes B2, C1, C2, D1, D2, and E1. Specific antibodies and GFP-promoter constructs show that genes B2, D1, D2, and E1 are exclusively expressed in intestinal cells. Using RNA interference (RNAi) by microinjection at 25 °C rather than at 20 °C we observe for the first time lethal phenotypes for C1 and D2. RNAi at 25 °C also shows that the known A1 phenotype occurs already in the late embryo after microinjection and is also observed by feeding which was not the case at 20 °C. Thus, RNAi at 25 °C may also be useful for the future analysis of other nematode genes. Finally, we show that triple RNAi at 20 °C is necessary for the combinations B2, D1, E1 and B2, D1, D2 to obtain a phenotype. Together with earlier results on genes A1, A2, A3, B1, and C2 RNAi phenotypes are now established for all 11 IF genes except for the A4 gene. RNAi phenotypes except for A2 (early larval lethality) and C2 (adult phenotype) relate to the late embryo. We conclude that in *C. elegans* cytoplasmic IFs are required for tissue integrity including late embryonic stages. This is in strong contrast to the mouse, where ablation of IF genes apparently does not affect the embryo proper.

Introduction

Most metazoan cells contain three major cytoskeletal polymers: actin, microtubules and intermediate filaments (IFs). IF proteins are highly conserved during evolution (Fuchs and Weber, 1994; Erber et al., 1998). Two common traits define the members of this diverse family. First, all exhibit a typical tripartite organization. A central domain, highly conserved in length is dominated by α-helices. This rod domain features highly conserved signature motifs at its N- and C-terminal ends. It is flanked by non-helical head and tail domains, which can vary greatly in length and primary sequence. Second, all cytoplasmic IFs can assemble into filaments, which are typically about 10 nm thick. The highly conserved rod domain plays a central role in this assembly process (for review see (Fuchs and Weber, 1994; Parry and Steinert, 1995; Herrmann et al., 2003)).

In mammals there are about 70 different members in the IF family (Hesse et al., 2001, 2004) which are subdivided into five types on the basis of their sequences, gene structures, expression patterns, and biochemical properties. Type I and type II keratins are the largest subfamilies and give rise to the epithelial keratin filaments, which are based on obligatory heteromeric double-stranded coiled coils formed by a type I and a type II keratin. Type III covers four mesenchymally expressed proteins which at least in vitro are able to form homopolymeric IF. Type IV spans mainly neuronal IF proteins while the type V covers the nuclear IF proteins, the lamins (for review see (Fuchs and Weber, 1994; Parry and Steinert, 1995; Herrmann et al., 2003)).

Orthologs of type I, II and III genes extend from the vertebrates to the cephalochordate *Branchiostoma* (Karabinos et al., 2002b) and several urochordates (Wang et al., 2002; Karabinos et al., 2004). Neurofilament type IV proteins

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emerged probably with the vertebrate lineage while the lower chordates have also some IF proteins without an obvious vertebrate counterpart. In contrast, all cytoplasmic IF proteins of protostomic animals relate more closely to the nuclear lamins. They have the long coil 1b domain of lamins and often display in addition a lamin homology segment in their tail domains (Weber et al., 1989; Erber et al., 1998).

Mutations in at least 16 human epidermal keratin genes cause various fragility syndromes of the skin (Irvine and McLean, 1999) and indicate that one function of IFs is connected with cellular resistance to mechanical stress. In addition, they may function as a scaffold allowing specific binding of some cellular constituents (Ku et al., 1998; Coulombe and Omary, 2002). Ablation of murine genes in general supports this view although in some cases the phenotypes may be rather weak (see (Hermann et al., 2003; Goldman, 2001)). Among the many murine knockout experiments, embryonic lethality only emerges in the case of double knockouts of either keratins 18 plus 19 (Hesse et al., 2000) or keratins 8 plus 19 (Tamai et al., 2000). Since the *Drosophila* genome lacks genes for cytoplasmic IF proteins (Goldstein and Gunawardena, 2000) the general function of these proteins was questioned (Hynes and Zhao, 2000). However, using (dsRNA)-mediated interference (RNAi) (Fire et al., 1998) on the nematode *C. elegans* allowed us to identify 4 IF genes necessary for development (Karabinos et al., 2001).

C. elegans has 11 genes encoding cytoplasmic IF proteins (A1 to A4, B1, B2, C1, C2, D1, D2, and E1). Some of these genes give rise to alternative splice variants (Dodemont et al., 1994; Karabinos et al., 2001; Woo et al., 2004). Previously, using RNAi at 20 °C by microinjection on all 11 IF genes we observed strong phenotypes for the genes A1, A2, A3, and B1; and a weak phenotype for C2. The phenotypes range from embryonic lethality (B1) and embryonic/larval lethality (A3) to larval lethality (A1 and A2). Using the RNAi feeding procedure (Timmons et al., 2001) at 20 °C phenotypes for B1 and A2 are recorded, and the adult C2 phenotype becomes more severe (Karabinos et al., 2003). The lack of phenotypes for A1 and A3 upon feeding agrees with the view that RNAi delivery by feeding may lose some early phenotypes but enhance some late phenotypes (Timmons et al., 2001).

Phenotypes A2, A3 and B1 involve displaced body muscles and paralysis. Since all three proteins are expressed in the hypodermis, their phenotypes arise by the reduction of hypodermal IF which participate in the transmission of force from the muscle cells to the cuticle (Karabinos et al., 2001, 2003). Similar results were recently obtained by mutant IF genes and/or by RNAi experiments for A2 and A3 (Hapiak et al., 2003), A3 and B1 (Woo et al., 2004) as well as for A2, B1 and C2 (Kamath et al., 2003) genes. Thus, at least five IF genes are essential for *C. elegans* development.

The spatial and temporal pattern of expression of the five essential cytoplasmic IF proteins A1, A2, A3, B1, C2 and the two IF proteins A4 and B2 was determined using GFP-promoter reporters and/or specific antibodies (Karabinos et al., 2001, 2002a, 2003). These analyses revealed the existence of two alternatively spliced variants for A1 (A1a and A1b) and B1 (B1a and B1b) genes (Karabinos et al., 2003). Interestingly, reduction of B1a by mutation or RNAi causes epidermal fragility, abnormal epidermal morphogenesis and muscle detachment while RNAi of B1b causes morphogenetic defects and defective outgrowth of the excretory cells (Woo et al., 2004). We found that one or both splice variants of the B1 gene are always co-expressed in a tissue-specific manner with at least one member of the A family in hypodermis, pharynx, pharyngeal-intestinal valve, excretory cells, uterus, vulva, and rectum (Karabinos et al., 2001, 2003). Using recombinant proteins we defined a keratin-like obligatory heteropolymer system by IF assembly in vitro in which B1 can form IFs when mixed with any members of the A subfamily and showed by RNAi that this system also holds in vivo. Proteins A1 and B1 have a similar and rather slow recovery in photobleaching experiments of the pharynx tonofilaments (Karabinos et al., 2003). Finally, using an anti-C2 antibody we reported that C2 is expressed in the cytoplasm and apical junctions of intestinal cells and in the pharynx apical junction (Karabinos et al., 2002a) while protein B2 is exclusively detected in the intestinal cells by the monoclonal anti-B2 antibody MH33 (Francis and Waterston, 1991; Karabinos et al., 2001; Segbert et al., 2004; Bossinger et al., 2004). After B2 RNAi no phenotype is observed (Karabinos et al., 2001; Bossinger et al., 2004) although immunoreactivity of B2 is abolished (Bossinger et al., 2004).

In the present study, we extended our analysis on the expression and function of the *C. elegans* IF proteins with special emphasis on proteins A4, B2, C1, D1, D2, and E1 which thus far did not yield any obvious RNAi phenotypes by the standard protocols (Karabinos et al., 2001, 2003; Kamath et al., 2003). We report that the IF proteins B2, D1, D2, and E1 are exclusively expressed in the intestine. Using RNAi at 25 °C rather than at 20 °C we observe for the first time embryonic lethality and early larval arrest for genes C1 and D2. In addition, simultaneous inactivation of genes B2, D1 and E1, which individually are not affected by RNAi, gives rise to an embryonic and postembryonic phenotype. Thus all *C. elegans* IF genes, except the A4 gene, are connected to RNAi phenotypes.

Materials and methods

Nucleic acid techniques

C. elegans strain N2 Bristol was cultured and harvested as described (Sulston and Hodgkin, 1988). The B2-, C1-, D1-, D2- and E1-promoter/gfp constructs were prepared from DNA fragments amplified by PCR on genomic DNA (Karabinos et al., 2001). The amplified B2 (1975 nt), C1 (1260 nt), D1 (1782 nt), D2 (2213 nt) and E1 (2704 nt) PCR products were ligated into the pEGFP-1 vector (Clontech, Heidelberg, Germany) as *Hin*dIII/*Bam*HI (B2) and *Xho*I/*Bam*HI, (C1, D1, D2, E1) fragments. The following primers were used: B2-promoter sense 5′-GAAATGT-CAAAAAGCT TCCATAGGGAAATCGTGTTATC-3′, antisense 5′-GTGCATCGAATAACTAACCGCGGATCC GATGAAGTCGC-TAA-3′; C1-promoter sense 5′-CTAAGAACTAGTAGGGCCTC-GAGCATAG CCTAGCATTTAGC-3′, antisense 5′-CGGAATAC-CTCCGTACAAGGATCCGACTGAAAATG AAAAATC-3′; D1-promoter sense 5′-GTTTTGCACTGAAAGCACCGCTCGAGTGG-CCGAT TGGTTAACGTTTAC-3′, antisense 5′-CTACACGAG-GGTTGAGTTGGATCCTTTTTTAAAGC CTGGAATC-3′; D2-promoter sense 5′-CAAGTTACCAACTGTTCATCTCGAGCTTT-GAGC TGTATAGAAG-3′, antisense 5′-GCGTTGGGTTGA-GAGGGTGGATCCTGGCGTTTTTATT CTAG-3′; E1-promoter sense 5′-GTTTTCAGAAATTGATAGCCTCGAGTTTTACTTGAA TAATTTG-3′, antisense 5′-CCCTAGCGTTAGCGGAATGGAT-CCGCTGAAACAGTAACCA TAAG-3′. The B2-, C1- and D2-promoter/cDNA/gfp constructs were prepared by cloning the *Bam*HI-, *Bam*HI- and *Age*I-digested coding regions of the B2, C1 and D2 cDNAs, respectively, (Karabinos et al., 2001) into the *Bam*HI-digested B2- and C1-promoter/gfp reporters and the AgeI-digested D2-promoter/gfp

reporters (see above). The coding regions were amplified using the following primers: B2-cDNA sense 5′-GTTTAACTTTAAGAAGGATCCGCCCTTCATATG TCG-3′, antisense 5′-CTTCGAATTGCCCTTTGGATCCGAAGAAGCGACCGTC-3′; C1-cDNA sense 5′-CTTTAAGAAGGAGGGATCCATATGTCCTTGTACG-3′, antisense 5′-GAATTCGCC CTTCTAGGGATCCGACGAAGAGTAGGAG-3′; D2-cDNA sense 5′-CGGAATTATCGATAA ACCGGTTTAAACCATGGC-3′, antisense 5′-CTAGAGGATCCCCGACCGGTGAGCTCGGA TCCAC-3′. Microinjection or ballistic transformation of the B2-, C1-, D1-, D2-, and E1-promoter/gfp plasmids as well as the B2-, C1- and D2-promoter/cDNA/gfp plasmids were used to make the unintegrated transgenic lines (Karabinos et al., 2001). These lines were used for the characterization of GFP expression.

Cloning of the B2, C1, D1, D2, and E1 cDNAs and in vitro dsRNA preparation, used for RNAi microinjection experiments, were as described (Karabinos et al., 2001). For RNAi feeding experiments, the cDNAs for A4, B2, C1, D1, D2, and E1 (Karabinos et al., 2001) were ligated between the phage T7 promoters of the L4440 feeding vector (Fraser et al., 2000). RNAi experiments by feeding were essentially as described (Karabinos et al., 2003).

Protein and antibody techniques

Cloning, expression and purification of the recombinant IF proteins A1b, A2, A3, A4, B1a, B2, C1, C2a, D1, D2, and E1 were reported (Karabinos et al., 2001; 2003). Rabbit antisera were raised with the following synthetic peptides as antigens (see Fig. 1): anti-B2 against the B2 peptide CIEKRSHIQTTVASSR; anti-C1 against the C1 peptide CSGDISAAGRHHESSY; anti-D2 against the D2 peptide CTSYHAYGSAYNDSLL; anti-E1 against the E1 peptide CWFVYTSNTEIGDADH. Prior to immunization the peptides were conjugated with hemocyanin via their extra N-terminal cysteine. All antisera were affinity purified on the corresponding peptides coupled to cyanogen bromide-activated Sepharose beads (Pharmacia, Uppsala, Sweden). The specificity of each antibody was verified by immunoblotting on purified recombinant IF proteins and on a total *C. elegans* protein extract as described (Karabinos et al., 2001). Indirect immunofluorescence microscopy of the *C. elegans* embryos and larvae was performed essentially as described (Karabinos et al., 2001, 2003). The anti-B2 antibody was diluted 1 to 50, the anti-C1 antibody 1 to 5, the anti-D2 antibody 1 to 50, the anti-E1 antibody 1 to 10, and the murine monoclonal anti-B2 antibody MH33 (Developmental Studies Hybridoma Bank, University Iowa, USA) 1 to 50.

RNA interference mediated by microinjection and feeding

RNAi experiments using the feeding or the microinjection techniques were essentially as described (Karabinos et al., 2001, 2003). RNAi experiments using the feeding plus microinjection techniques combined and modified the original Fraser et al. (2000) and Fire et al. (1998) protocols as follows. The 5-cm NGM plates containing carbenicillin (25 μg/ml) and 1 mM IPTG were inoculated with 5 μl of the B2-, D1-, D2- or E1-cDNA/L4440-transformed HT115(DE3) bacterial overnight culture, or their double or triple mixtures, and left for six hours at room temperature. The adult hermaphrodites (P0) were washed with M9 medium and placed on the feeding plates and incubated for 72 hours at 20 °C. The adult progeny (F1) was used for microinjection of the corresponding dsRNA(s) as described (Karabinos et al., 2001). Injected hermaphrodites were transferred to the corresponding NGM feeding plates (see above) and their progeny (F2) growing at 20 °C was scored for abnormalities by Nomarski microscopy.

Results

Immunoblot analysis of polyclonal IF antibodies

To determine the expression patterns of the B2, C1, D1, D2 and E1 proteins we raised five peptide antibodies (Fig. 1) in rabbits and purified them by affinity chromatography (see Materials and methods for details). The specificity of the antibodies was demonstrated by immunoblot analyses on eleven recombinant nematode IF proteins (Karabinos et al., 2001, 2003) and on a total *C. elegans* protein extract (Fig. 2). As shown in Figure 2A, the anti-B2, -C1, -D2, and -E1 antibodies recognized exclusively the corresponding recombinant protein while the anti-D1 antibody displayed a number of strong cross-reactions (not shown) and was removed from this study.

In the total protein extract of *C. elegans* the anti-B2 antibody recognized a double band at approximately 60 – 62 kDa which probably reflects the two alternatively spliced variants of the B2 gene (B2-H, 61695 Da; B2-L, 59856 Da) (Dodemont et al., 1994). The lack of reactivity of the anti-C1 antibody on immunoblotting of the total protein extract (Fig. 2) is most likely due to the small amount of C1 present in such extracts. The D2-specific antibody recognized a polypeptide of approximately 50 kDa, which fits the calculated molecular mass from the D2 sequence (D2, 51580 Da) (Karabinos et al., 2001). Finally, the E1 antibody decorated in the total protein extract two polypeptides of approximately 90 kDa and 58 kDa. The higher mass is in good agreement with that calculated from the E1 sequence (89050 Da) while the smaller polypeptide (marked with the asterisk in Fig. 2B) might represent a splice variant lacking in part the central region of the long E1 tail sequence (Fig. 1) (Karabinos et al., 2001). Results from the recent genomic analysis of *Caenorhabditis briggsae* (Stein et al., 2003) indicating the existence of two isoforms derived from the E1 gene (CBP18554, CBP18555; www.wormbase.org/db/seq/gbrowse/briggsae) support this view.

We also analyzed the specificity of the anti-C2 antibody described previously (Karabinos et al., 2002a). In contrast to previous results documenting only a single C2-positive polypeptide of about 55 kDa (Karabinos et al. 2002a), we now find with a more concentrated antibody solution an additional C2-positive band of about 66 kDa (marked with the asterisk in Fig. 2B). This result supports the existence of two splice variants C2-L and C2-H of the C2 gene (calculated protein molecular weights 56207 and 70101 Da, respectively), as predicted by Dodemont et al. (1994). We renamed these two C2 splice variants C2a and C2b in agreement with the nomenclature used on other IF genes (Karabinos et al., 2001, 2003).

Tissue-specific expression of IF proteins B2, C1, D1, D2, and E1

Immunolocalization of proteins B2 and D2 was performed using the B2-specific polyclonal antibody (see Fig. 2), the B2-specific monoclonal antibody MH33 (Karabinos et al., 2001; Bossinger et al., 2004) and the polyclonal anti-D2 antibody (see Fig. 2). As shown in Figure 3A – C for B2, expression of both proteins is exclusively seen in the terminal web of the intestinal cells of the embryo starting in the bean developmental stage. The same expression pattern of both proteins was also detected in all larval developmental stages and in adults as documented by the double staining experiment shown in Figure 3D – I. The transformation experiments with the B2- and D2-promoter/gfp reporters (data not shown) as well as with the B2- and D2-promoter/cDNA/gfp reporters (Fig. 3J – M) fully confirmed these results.

Double staining experiments with the C1-specific rabbit antibody (see Fig. 2) and the monoclonal antibody MH4, which strongly reacted with recombinant IF proteins A1, A2 and A3

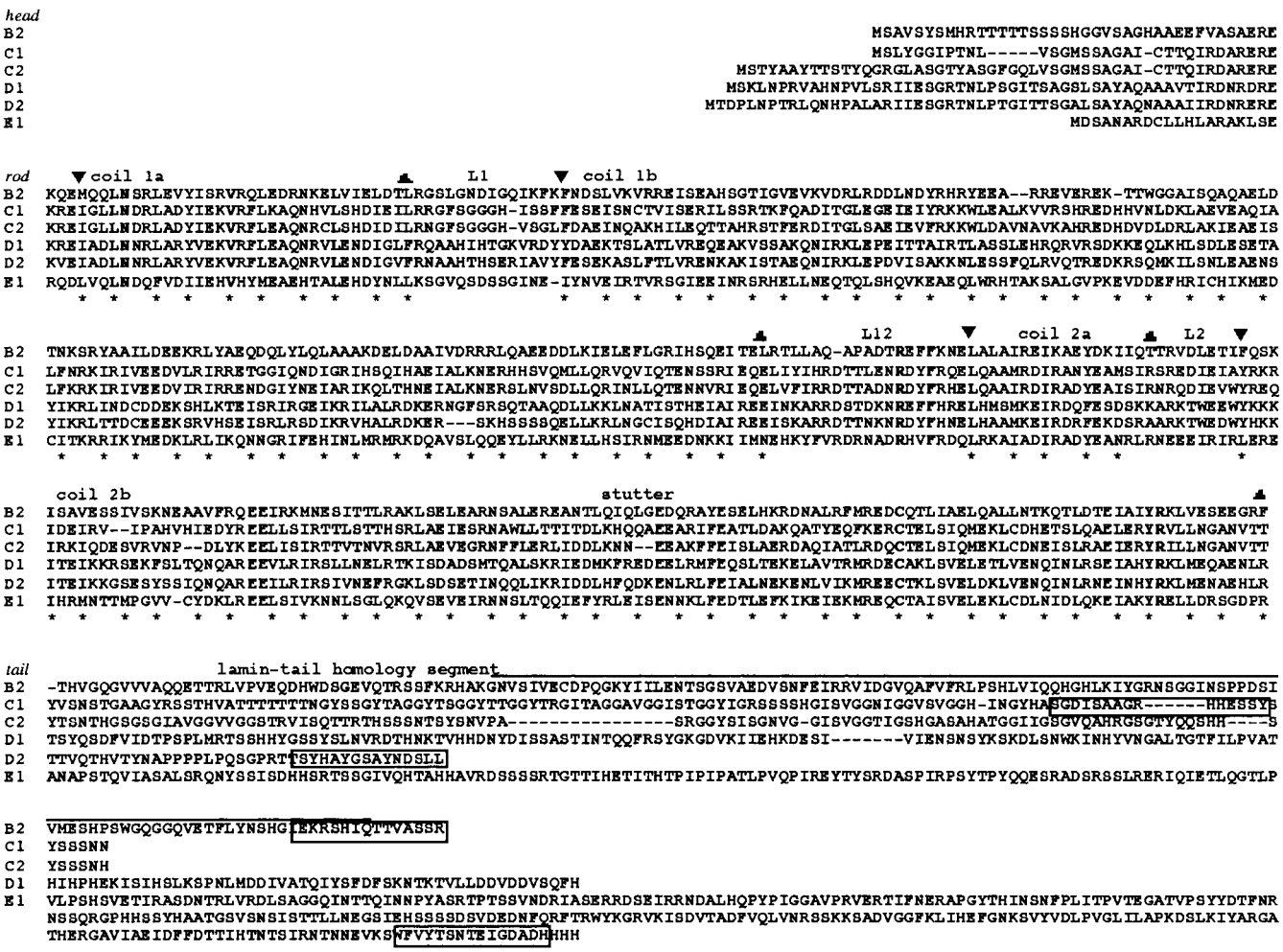

Fig. 1. Sequence alignment of the six *C. elegans* IF proteins expressed in the intestine. The three-domain structure of the typical IF protein (head, rod, tail) is indicated. The ends of the rod subdomains (coils 1a, 1b, 2a and 2b) are marked by arrowheads above the amino acid sequences. Asterisks (*) given below the amino acid sequences mark *a* and *d* positions of the heptad repeat pattern with the stutter in coil 2b (for details on IF structure see (Fuchs and Weber, 1994; Parry and Steinert, 1995; Strelkov et al., 2003)). Dashes are used to optimize the sequence alignment. Bold letters indicate residues in the rod domains that are identical in all six proteins. Sequences of synthetic peptides used for antibody production are boxed.

in immunoblots (Karabinos et al., 2001), are provided in Figure 4. These experiments showed coexpression of the C1 and the MH4 antigens in the dorsal and ventral hypodermis of the bean (Fig. 4A – D), 1.5-fold (Fig. 4E, F) and 3-fold (Fig. 4G, H) embryos, in the larvae (Fig. 4I – K) and in adults (not shown). However, in contrast to the MH4 staining pattern (marked by the arrow in Fig. 4J) no visible C1 expression was seen in the pharynx and also the intensity of C1 staining in the larval and adult hypodermis was significantly weaker. C1 expression was detected weakly in the intestinal terminal web of the embryos (not shown) and strongly in the same structure of all larval developmental stages (Fig. 4K) and of the adults (not shown). The expression of C1 in the intestinal terminal web and in the hypodermis was also observed in worms transformed with the C1-promoter/cDNA/gfp reporter (Fig. 4L – N; for details see Materials and methods). Moreover, the C1-GFP expression was also detected in cellular junctions of the pharynx in the early larval developmental stage (Fig. 4L – P).

Finally, the expression patterns of genes D1 and E1 were determined using the corresponding D1- and E1-promoter/gfp reporters and the E1-specific antibody. As shown in Figure 5A – D the D1-promoter/gfp reporter was exclusively detected in the intestine of late embryos, larvae and adults. The same holds also for the E1-promoter/gfp-expressing worms (Fig. 5G – J). Immunofluorescence experiments using the E1-specific antibody showed strong staining of the intestine (Fig. 5E). An additional staining in some non-identified pharyngeal structure of embryos, larvae and adult (marked by the arrow in Fig. 5E) is currently not understood.

RNAi analysis by microinjection for the 11 *C. elegans* IF genes at 25 °C

Since our previous RNAi experiments on B2, C1, C2, D1, D2, and E1 by microinjection and feeding at the standard temperature of 20 °C did not reveal any phenotypes (Karabinos et al., 2001, 2003) we decided to repeat the experiments at 25 °C. Using these conditions, we observed strong phenotypes for the C1, C2 and D2 genes but no phenotypes for genes B2, D1 and E1 (Tables 1 and 2).

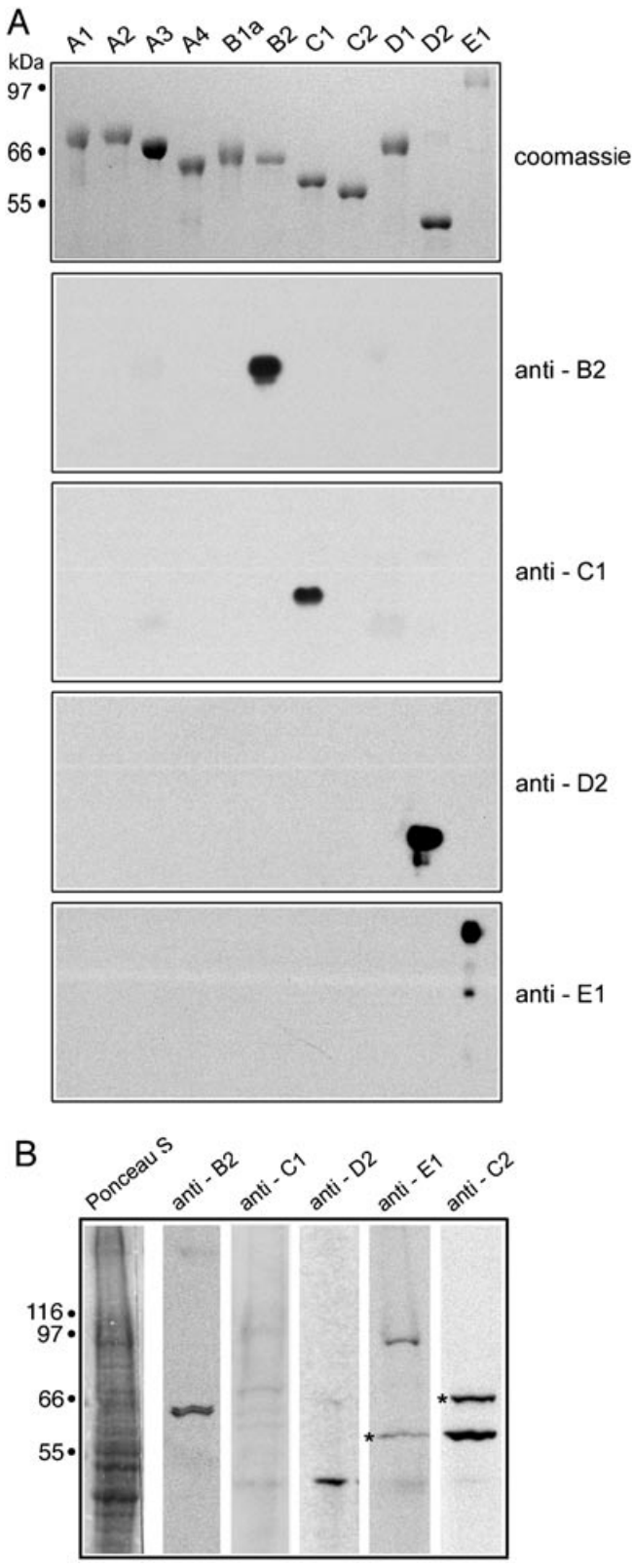

Fig. 2. Immunoblot analyses of polyclonal rabbit antibodies to proteins B2, C1, C2, D2, and E1. (A) Approximately equal amounts of recombinant IF proteins A1b, A2, A3, A4, B1a, B2, C1, C2a, D1, D2, and E1 (Karabinos et al., 2003) were separated by SDS-PAGE and stained with Coomassie or blotted onto nitrocellulose membranes. Blots were incubated with affinity-purified rabbit anti-B2, -C1, -D2, and -E1 antibodies as indicated on the right of the panels. All four antibodies recognized the corresponding recombinant protein. Molecular mass standards (in kDa) are given on the left of the upper panel. (B) Equal amounts of *C. elegans* total protein extract were separated by SDS-PAGE, blotted onto nitrocellulose and stained with Ponceau S or incubated with affinity-purified anti-B2, -C1, -C2, -D2, and -E1 antibodies as indicated above the individual blot strips. The anti-B2 antibody recognized a double band at approximately 60–62 kDa (see text). The lack of reactivity of the anti-C1 antibody in the total protein extract is most likely due to the small amount of C1 present in the extract. The anti-D2 antibody recognized a single polypeptide of approximately 50 kDa while the anti-E1 antibody decorated in the total protein extract two polypeptides of approximately 90 and 58 kDa (see text). The anti-C2 antibody (Karabinos et al., 2002a) revealed two polypeptides of approximate molecular masses of 55 kDa and 66 kDa (marked with an asterisk) supporting the existence of the two splice variants arising from the C2 gene (see text for details). Molecular mass standards (in kDa) are given on the left.

The C1 RNAi/25 °C phenotype was characterized by late embryonic lethality (7% compared to 2.5% of wild type at 25 °C) and early larval arrest (16% compared to 1.5% of wild type at 25 °C). The arrested larvae exhibit abnormal epidermal morphology mostly in the head region and occasionally muscle detachment defects throughout the body (Fig. 6). The C2 RNAi/25 °C phenotype was characterized by adult lethality (16% compared to 2% of wild type at 25 °C) mostly due to rupture of the vulva and/or anus. Interestingly, a similar phenotype but with much lower penetrance (3%) was previously observed in the C2 RNAi feeding experiment at 20 °C (Karabinos et al., 2003; Kamath et al., 2003). Finally, the D2 RNAi/25 °C phenotype revealed late embryonic lethality in about 10% of the animals and an increased early larval arrest (4% compared to 1.5% of wild type at 25 °C). Two of five arrested larvae displayed abnormal intestine morphology (Fig. 6E). Some parts of the intestine were thicker (marked with the arrowhead in Fig. 6E), and we were unable to identify an intestinal lumen in these regions (marked with arrows in Fig. 6E).

The temperature-dependent changes in the RNAi phenotypes found for genes C1, C2 and D2 (see above) invited use of the method on the five other *C. elegans* IF genes (A1, A2, A3, A4, and B1). As shown in Table 1, microinjection and 25 °C shifted the A1 phenotype to an earlier developmental stage and induced a higher penetrance of the known A2 and A3 phenotypes. Thus, in the A1 RNAi/25 °C experiments we observed late embryonic lethality of 30% of the A1 RNAi animals in addition to the early larval arrest exclusively seen at 20 °C (Karabinos et al., 2001). In the A2 RNAi/25 °C experiments we found an increase of early larval lethality from 85% (20 °C; Karabinos et al., 2001; Hapiak et al., 2003) to 100% (25 °C). In the A3 RNAi/25 °C experiments we observed an increase of the late embryonic lethality from 25% (20 °C; Karabinos et al., 2001; Hapiak et al., 2003) to 75% (25 °C; Table 1). The previously reported late embryonic lethal B1 phenotype at 20 °C (Karabinos et al., 2001; Woo et al., 2004) remained unchanged at 25 °C. No phenotype was detected for the A4 gene at 25 °C (Table 1).

RNAi analysis of the 11 *C. elegans* IF genes by feeding at 25 °C

The RNAi feeding delivery method can be less effective than microinjection in establishing early developmental RNAi

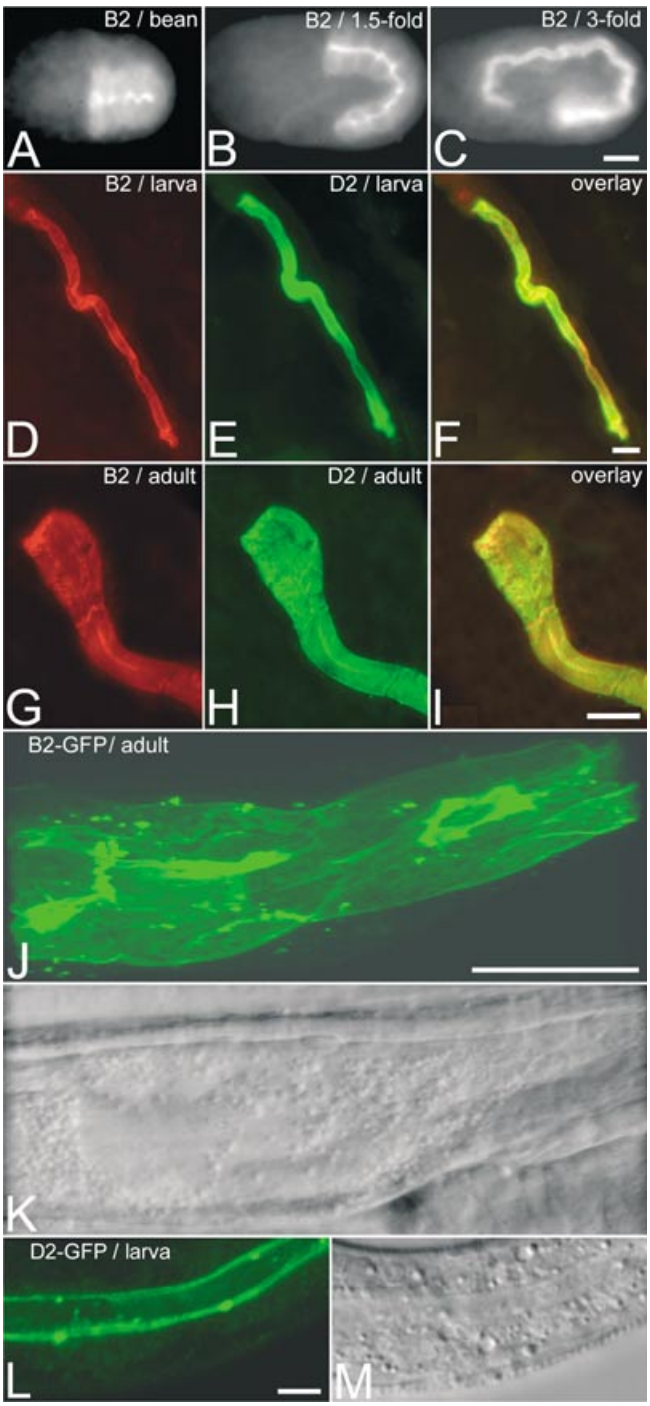

Fig. 3. Tissue-specific expression of proteins B2 and D2. Expression of B2 protein, determined by monoclonal antibody MH33, is exclusively seen in the intestinal terminal web of embryos (A – C), larva (D) and adult (G). This holds also for protein D2, stained in the same specimens (E, H) with the polyclonal anti-D2 antibody. Overlays of B2 (MH33) and D2 staining are shown in (F, I). The B2-promoter/cDNA/gfp (J) and the D2-promoter/cDNA/gfp (L) constructs localized the B2-GFP and D2-GFP to the intestinal terminal web. Nomarski phase contrast (K, M) was used to facilitate the identification of cells and tissues. Scale bars represent 10 μm for (A – C); 25 μm for D – F, G – I, J, K); 5 μm for (L, M).

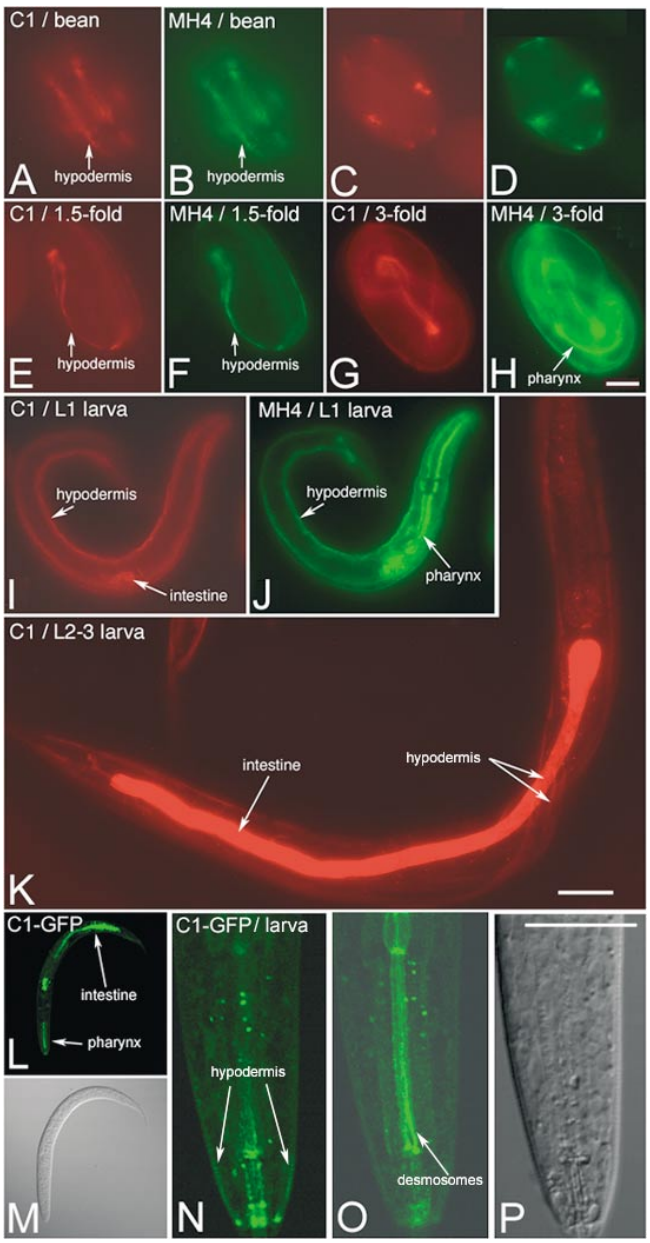

Fig. 4. Tissue-specific expression of C1 protein. Whole-mount embryos of the three developmental stages bean, 1.5-fold and 3-fold were double labeled with affinity-purified anti-C1 antibody and with the monoclonal anti-A1 to A3 IF antibody MH4. The C1 and MH4 antigens are coexpressed in the dorsal and ventral hypodermis of the bean (A – D), 1.5-fold (E and F) and 3-fold (G and H) embryos as indicated. The MH4 antibody decorates in addition the pharyngeal tonofilaments of the 3-fold embryo marked with the arrow (H). Whole-mount larvae of the two developmental stages L1 (I, J) and L2/3 (K) were labeled with affinity-purified anti-C1 antibody, and in the case of L1 larvae double-labeled with the monoclonal anti-A1 – A3 IF antibody MH4. The C1 and MH4 antigens are coexpressed in the hypodermis while only the anti-C1 antibody decorates the intestine of both larval developmental stages (I, K) and adults. The MH4 antibody decorates in addition the pharyngeal tonofilaments (marked with the arrow in (J)) and some other structures (for detailed MH4 expression pattern see (Francis and Waterston, 1991, Hresko et al., 1994; Karabinos et al., 2001, 2003)). The C1-promoter/cDNA/gfp transgenic larva showed C1-GFP expression in the intestinal terminal web (L), the hypodermis (N) and in cellular junctions of the pharynx (O). Nomarski phase contrast (M, P). Scale bars represent 10 μm for (A – J); 20 μm for (K); 15 μm for (L – P).

Table 1. RNAi by microinjection of the 11 *C. elegans* IF genes at 20 °C and 25 °C.

Genes	RNAi phenotype at 20 °C*	RNAi phenotype at 25 °C
A1	L1 arrest (100%)	Late embryonic lethality (30%), L1 arrest (70%), (n = 63)
A2	Early larval lethality (85%), late larval lethality (15%)	Early larval lethality (100%), (n = 90), excretory canals uneven, displaced body muscles, paralysis
A3	Late embryonic lethality (25%), early larval lethality (75%)	Late embryonic lethality (75%), early larval lethality (25%), (n = 48), excretory canals uneven, displaced body muscles, hypodermis detached from the cuticle, paralysis
A4	No phenotype	No phenotype
B1	Late embryonic lethality (100%)	Late embryonic lethality (100%), n = 72, from hypodermal to severe general morphological defects
B2	No phenotype	No phenotype
C1	No phenotype	Late embryonic lethality (7%), early larval arrest (16%), (n = 85), abnormal epidermal morphology localized mostly to the head region and occasional muscle detachment defects throughout the body
C2	Dumpy (10%)	Adult lethality (16%), (n = 55), rupture of vulva and/or anus
D1	No phenotype	No phenotype
D2	No phenotype	Late embryonic lethality (10%), early larval arrest (4%), (n = 140), morphological defects
E1	No phenotype	No phenotype

* For details see (Karabinos et al., 2001).

Table 2. RNAi by feeding of the 11 *C. elegans* IF genes at 20 °C and 25 °C.

Genes	RNAi phenotype at 20 °C*	RNAi phenotype at 25 °C
A1	No phenotype	Late embryonic lethality (10%), early larval arrest (13%), slowly growing larvae (13%), (n = 80)
A2	Late larval and adult paralysis (30%)	Late larval lethality (35%), (n = 120), excretory canals uneven, displaced body muscles, paralysis
A3	No phenotype	No phenotype
A4	No phenotype	No phenotype
B1	Late larval and adult paralysis (35%)	Late embryonic lethality (15%), early and late larval lethality (85%), (n = 145), excretory canals uneven, displaced body muscles, hypodermis detached from the cuticle, paralysis
B2	No phenotype	No phenotype
C1	No phenotype	Early larval arrest (10%), (n = 70), abnormal epidermal morphology localized mostly to the head region and occasional muscle detachment defects throughout the body
C2	Adult lethality (3%)	Adult lethality (10%), rupture of vulva and/or anus
D1	No phenotype	No phenotype
D2	No phenotype	No phenotype
E1	No phenotype	No phenotype

* The A1, A2, A3, B1, and C2 results at 20 °C were previously reported (Karabinos et al., 2003).

phenotypes, while it can be more effective than microinjection for late developmental stages (Timmons et al., 2001). In line with this prediction we previously found a developmental shift of the B1 phenotype from embryonic lethality to late larval/adult lethality, and similar changes have been observed in the A2 RNAi feeding experiment. Moreover, RNAi feeding at 20 °C did not detect the embryonic and early larval phenotypes of the A3 and A1 genes observed by microinjection. On the other side, the mild dumpy phenotype of RNAi-microinjected C2 animals (Karabinos et al., 2001) changed to adult lethality upon feeding (Table 2) (Karabinos et al., 2003; Kamath et al., 2003). No phenotype was detected in the RNAi feeding experiments at 20 °C for genes A4, B2, C1, D1, D2 and E1 (Table 2) (see also (Kamath et al., 2003)).

In contrast to the RNAi feeding experiments at 20 °C strong A1 and C1 RNAi feeding phenotypes were seen at 25 °C (Table 2). The A1 phenotype was characterized by late embryonic lethality (10%), early larval arrest (13%) and slow larval growth (13%). The C1 phenotype showed early larval arrest (10%) with epidermal defects and with muscle detachments (Fig. 6; Table 2). In addition, the B1 RNAi feeding phenotype at 20 °C involving late larval lethality (Karabinos et al., 2003; Kamath et al., 2003) was shifted at 25 °C to embryonic lethality (15%). The 3%-penetrance of the adult lethal C2 phenotype at 20 °C (Karabinos et al., 2003; Kamath et al., 2003) was shifted to 10% at 25 °C. Finally, no phenotypes were detected for the A4, B2, D1, D2, and E1 genes by feeding at 25 °C.

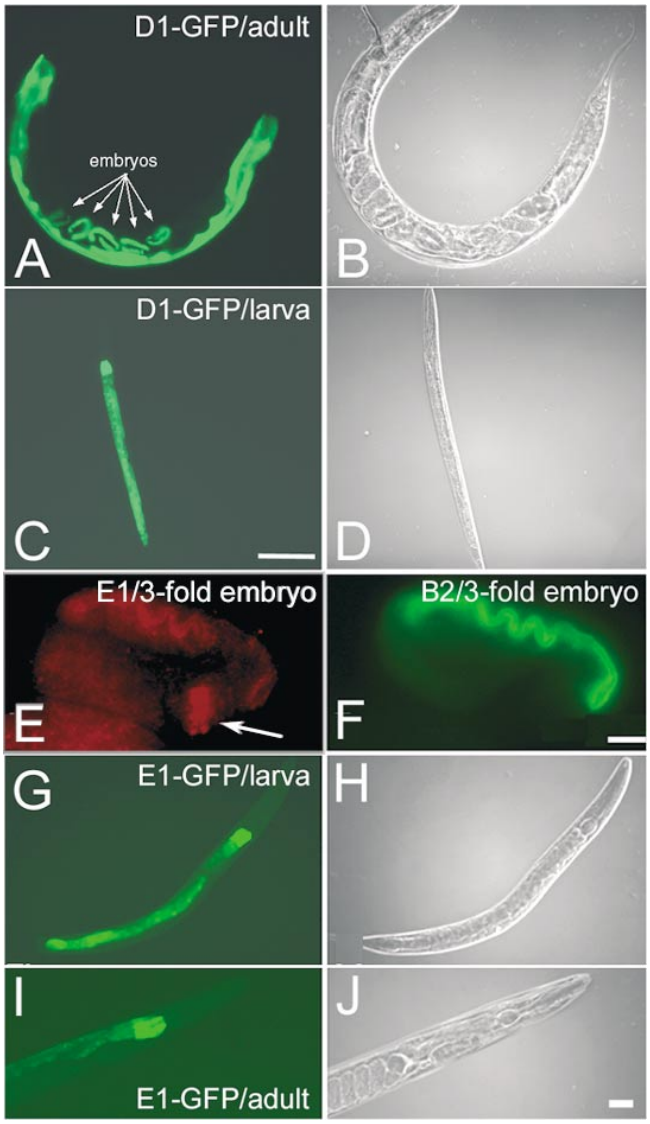

Fig. 5. Tissue-specific expression of the D1- and E1-promoter/gfp reporter and E1 protein. Expression of D1-promoter driven GFP in the adult, embryos (A, B) and the larva (C, D) was detected exclusively in the intestine. Double staining with the affinity-purified anti-E1 antibody (E) and the monoclonal anti-B2 antibody MH33 (F) revealed coexpression of E1 and B2 in the intestinal terminal web of the embryo. In addition, the anti-E1 antibody detects some unidentified structures in the pharynx (marked with the arrow in (E)). Expression of the E1-promoter driven GFP was detected exclusively in the intestine of larvae (G, H) and adults (I, J). Phase contrast (B, D, H, J) was used to facilitate the identification of cells and tissues. Scale bars represent 100 μm for (A-D); 10 μm for (E, F); 50 μm for (G, J).

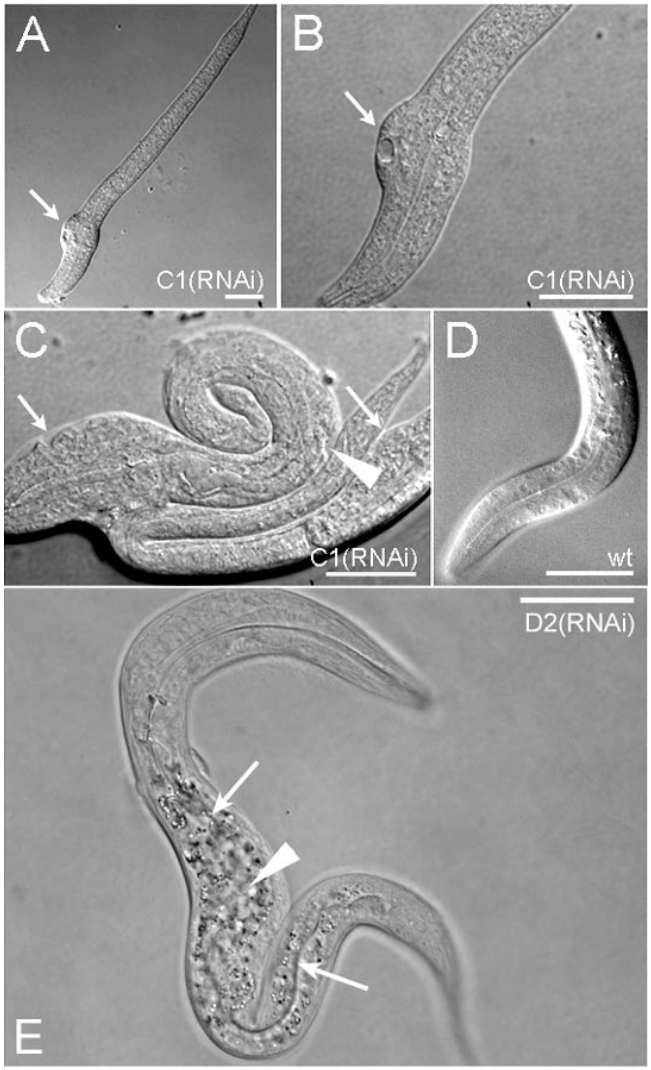

Fig. 6. Postembryonic phenotypes of C1 and D2 RNAi at 25 °C. Hypodermal defects of early larvae induced by C1 RNAi microinjection at 25 °C (A – D). Nomarski images of the early larvae document abnormal epidermal morphology in the head regions (marked by arrows in (A – C)) and muscle detachment defects throughout the body (marked with the arrowhead in (C)). (B) is a higher magnification of (A). (D) Nomarski image of a wild-type L1 larva. The early D2 RNAi larva (E) displayed areas of abnormal intestinal morphology (arrowhead) with nonvisible lumen (arrows). Scale bars represent 25 μm.

Double and triple RNAi experiments by microinjection plus feeding reveal essential functions of the B2, D1 and E1 genes in worm development

Of the four IF genes (B2, D1, D2, and E1), which are exclusively expressed in the intestine, only D2 provided a detectable RNAi phenotype (microinjection and 25 °C; see Tables 1 and 2). To investigate possible functions of the three remaining genes we treated worms with double or triple mixtures of B2, D1, and E1 dsRNAs, which were delivered by the feeding plus microinjection protocol at 20 °C (see Materials and methods for details). Of all possible mixtures of B2, D1, and E1 dsRNAs, the triple RNAi experiments revealed the strongest phenotype. It involved embryonic lethality (15%), slowly growing larvae (10%) and smaller adults (5%) for the 110 animals scored. The B2/D1/E1 (RNAi) embryos arrested at the 2 – 3-fold stage, had visible vacuoles, mostly localized in the posterior region, and often displayed ruptures (Fig. 7A). The smaller adults were about 0.7 mm in length, had unusually high numbers of undifferentiated germ cells in the last part of the ovary (marked with arrows in Fig. 7E) and showed a reduced brood size (40 – 80 compared to about 300 for the wild type). Interestingly, the penetrance of this phenotype was significantly increased when E1 dsRNA (but not B2 or D1 dsRNAs) was replaced by D2 dsRNA. In this triple RNAi experiment we observed lethality

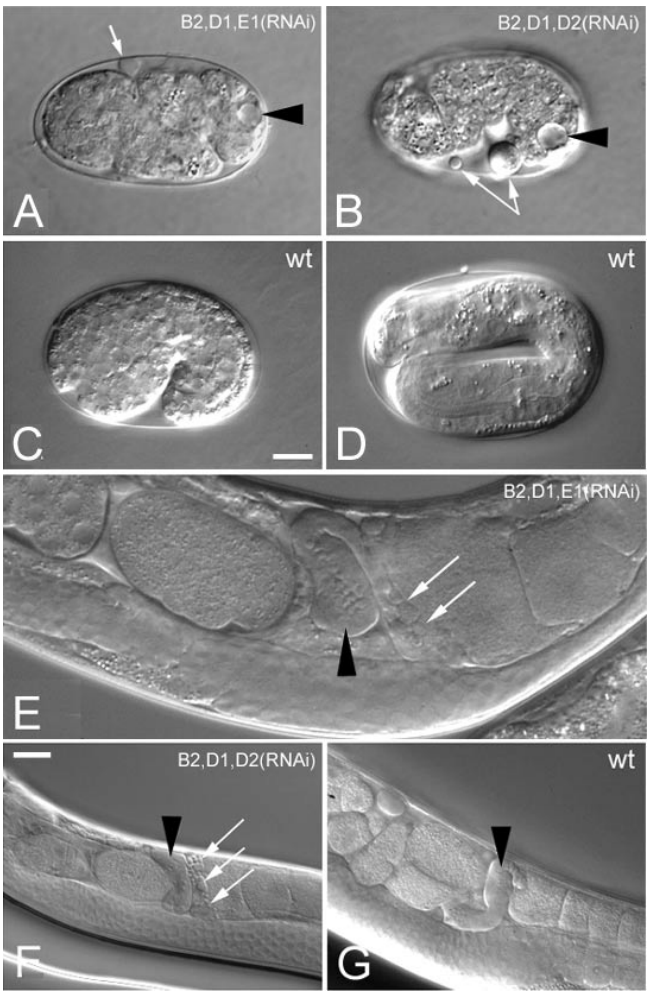

Fig. 7. Embryonic and postembryonic defects of B2, D1, E1 and B2, D1, D2 (triple RNAi) animals. The B2, D1, E1 (A) and B2, D1, D2 (B) embryos show ruptures resulting in loss of internal contents (white arrows) and vacuoles (black arrowheads). For comparison, 1.5-fold (C) and 3-fold (D) stage wild-type embryos are shown. The B2, D1, E1 (E) and B2, D1, D2 (F) (triple RNAi) adults are smaller than the wild type and display a higher number of undifferentiated germ cells in the ovary (white arrows) just before the spermatheca (black arrowheads). (G) Wild-type gonad with the spermatheca (black arrowhead). Scale bars represent 10 μm for (A-E); 20 μm for (F, G).

of 31% of the embryos (Fig. 7B), slow growth of 20% of the scored larvae as well as 8% smaller adults (Fig. 7F). We note that at 20 °C none of the 3 genes is affected by single RNAi.

Discussion

Using specific antibodies and GFP reporters we previously analyzed the expression patterns of six (A1, A2, A3, A4, B1, and C2) of the 11 IF proteins present in *C. elegans* (Karabinos et al., 2001, 2002a, 2003). This study outlines the developmental control of expression for the five remaining IF proteins (B2, C1, D1, D2, and E1). Immunofluorescence microscopy and/or the transgenic promoter-driven GFP-expressing worms reveal coexpression of proteins B2, D1, D2, and E1 exclusively in the intestinal terminal web. These patterns are seen already in embryos and persist in all larval stages and the adults. In contrast, protein C1 shows some interesting changes in expression during development. Protein C1 is strongly expressed in the hypodermis and weakly in the intestine of the embryo while the opposite situation seems to hold in the larva and adult stages. Moreover, significant C1 expression was also seen in the pharyngeal apical junctions of the early larva stage. Thus, C1 colocalizes with the related protein C2 at apical junctions (Karabinos et al., 2002a). The hypodermal C1 expression pattern strongly resembles that detected with the monoclonal antibody MH4 which is specific for proteins A1, A2 and A3 (Karabinos et al., 2001). Thus, the four proteins B2, D1, D2, and E1 are expressed exclusively in the intestine, which shows additionally C1 (see above) and C2 (Karabinos et al., 2002a) at least during some developmental stages.

Previous functional analyses using the standard RNAi microinjection or feeding protocols at 20 °C showed strong phenotypes for A1, A2, A3, B1, and C2 genes and no phenotypes for genes A4, B2, C1, D1, D2, and E1 (Karabinos et al., 2001, 2003) (see also (Hapiak et al., 2003; Woo et al., 2004; Bossinger et al., 2004)). However, the RNAi analyses at 25 °C, presented here, provided strong phenotypes also for genes C1 and D2 (Fig. 6, Tables 1, 2) and shifted the known A1 phenotype achieved at 20 °C (Karabinos et al., 2001, 2003) from the early larva to the late embryo. Thus RNAi at 25 °C may also be useful for the analysis of other nematode genes.

The C1 RNAi/25 °C phenotype induced by microinjection was characterized by low penetrance late embryonic lethality (7%) and an early larval arrest (16%), while only the postembryonic phenotype was observed in experiments in which the C1 dsRNA was introduced by feeding. The morphological defects of the arrested C1 RNAi/25 °C larvae were mainly concentrated in the hypodermis of the head while occasionally muscle displacement was seen throughout the body muscle. This phenotype resembled the previously reported A2, A3 and B1 RNAi phenotypes achieved at 20 °C. However, the penetrance and intensity were much stronger in these 3 phenotypes (for details see (Karabinos et al., 2001, 2003; Hapiak et al., 2003; Woo et al., 2004)). Thus, protein C1 seems to be a component of the IF network responsible for the mechanical integrity of the hypodermis. Future studies using high-resolution immunoelectron microscopy may help to understand the function of C1 and its relation to the main B1/A3/A2 IF cytoskeleton of the hypodermis.

The second new RNAi/25 °C phenotype was induced by microinjection of dsRNA specific to gene D2. It comprises a low penetrance late embryonic lethality (10%) and an approximately 2.5-fold increase of larval arrest over the wild-type control at 25 °C (Tables 1 and 2). Two of five arrested larvae displayed abnormal intestinal morphology (Fig. 6E). Some parts of the intestine were thicker and an intestinal lumen could not be identified in these regions. It remains to be seen whether this phenotype reflects morphological or proliferation defects of the intestinal cells. Of the four IF genes expressed exclusively in the intestine only gene D2 gave rise to an RNAi phenotype which was observed only by the microinjection procedure and not by the feeding delivery protocol (Tables 1 and 2).

The exclusive expression of genes B2, D1, D2, and E1 in the intestine raised the question whether some IF proteins fulfilling the same or very similar functions can substitute for each other. This situation arose previously with mouse keratin genes expressed in interior epithelia. While single gene ablation provided no phenotype the simultaneous knockouts of 2 genes

revealed embryonic lethality (Hesse et al., 2000; Tamai et al., 2000). We therefore explored double and triple RNAi using a protocol based on microinjection plus feeding at 20 °C. The simultaneous inactivation of genes B2, D1 and E1 provided a stronger phenotype than double RNAi combinations. The B2/D1/E1 RNAi embryos arrested at the 2–3-fold stage with visible vacuoles and often also revealed tissue ruptures (Fig. 7A). The penetrance of the B2/D1/E1 phenotype was significantly increased when the E1 dsRNA in the mixture was replaced by D2 dsRNA.

The intestinal terminal web (known in nematodes as the endotube) is a dense cytoskeletal layer which underlies the apical microvilli of the epithelial cells and which is thought to contain an extensive IF network (Munn and Greenwood, 1984; Leung et al., 1999). The structure joins directly to the apical junctions (CeAJ), which are essential for integrity of adjacent intestinal cells. In addition, the CeAJ are sites of intensive intercellular signalling, and defects in their organization may result in apoptosis or uncontrolled cell division. There are two independent protein systems operating in CeAJ (for review see (Chin-Sang and Chisholm, 2000; Michaux et al., 2001; Knust and Bossinger, 2002)). The first comprises the E-cadherin, α-catenin and β-catenin homologues HMR-1, HMP-1 and HMP-2, while the other includes proteins AJM-1 and DLG-1. Mutations in the former protein system are lethal due to defects in the actin cytoskeleton and its anchoring (Costa et al., 1998), while disruption of the latter two proteins causes disappearance of the electron-dense apical structures and embryonic death (Köppen et al., 2001). Removal of HMP-1, AJM-1 or DLG-1 by RNAi has little effect on the overall position of the terminal web. In contrast, removal of the LET-413 protein, which is the homolog of the *Drosophila* Scribble protein needed for proper localization of DLG-1 and AJM-1 in CeAJ (Köppen et al., 2001; McMahon et al., 2001), leads to a basolateral expansion of the terminal web (Bossinger et al., 2004). Interestingly, the *let-413* embryos show vacuoles, ruptures and no discernible lumen in the intestine (Legouis et al., 2000) and thus resemble the D2 and B2/D1/E1 RNAi phenotypes described above. On the other side, the reduced body and brood size of the triple B2/D1/E1 knockdowns resemble the phenotype of the recently described *pep-2* mutant (Meissner et al., 2004). The nematode PEP-2 protein is expressed along the apical membrane of the intestinal cells and mediates the uptake of peptides into the intestine. PEP-2 was shown to cross-talk with both the *C. elegans* TOR and DAF/insulin signalling pathways which regulate cell growth, proliferation and aging in response to nutrients.

Although these results should help to define a model of how CeAJ/IF complexes assemble and maintain cell integrity and polarity (see also (Segbert et al., 2004)) the precise molecular picture will probably require double immunoelectron microscopical information of high resolution. It could define whether there is only one or more populations of IFs and define the role of the C2 protein, which by immunofluorescence seems present in IFs of the terminal web but also strongly enriched at CeAJ (Karabinos et al., 2002a; Segbert et al., 2004). Interestingly, there are at least 2 different C2 splice variants identified by cDNA cloning (Dodemont et al., 1994) and immunoblotting (Fig. 2B).

A better understanding of the intestinal IF can also be expected from in vitro filament assembly studies using recombinant proteins. Preliminary work indicates that the individual proteins B2, D1, D2, and E1 do not form IFs but yield only aggregates. In contrast, the mixtures of B2 and D2 seem to form IFs but electron micrographs indicate an inhomogenous product of IFs and thicker ribbons of filaments. A solution of this problem still requires a detailed analysis to define optimal assembly conditions.

The 11 *C. elegans* IF proteins seem to form 3 groups. The *first* IF group contains proteins A1a, A1b, A2, A3, A4, B1a, and B1b. In this "B1/A" system one or both splice variants of the B1 gene is coexpressed with at least one A protein in several epithelial organs including the hypodermis and the pharynx. B1 together with proteins A1, A2 or A3 forms long heterofilaments in vitro (Karabinos et al., 2003). Different RNAi and/or genetic mutant experiments of B1 (Karabinos et al., 2001; Woo et al., 2004), A1 (this study) and A3 (Karabinos et al., 2001; Hapiak et al., 2003; Woo et al., 2004) genes result in embryonic death in the 2–3-fold stage as well as in postembryonic defects in the hypodermis/muscle attachment, pharynx and excretory canals. Similar experiments using A2, the larval stage up-regulated IF gene (Karabinos et al., 2002a), show muscle detachment and larval lethality (Karabinos et al., 2001; Hapiak et al., 2003; Kamath et al., 2003) while no obvious RNAi phenotype was so far observed for A4, the last member of the A IF family (this study; Karabinos et al., 2001; Kamath et al., 2003). Thus, the B1/A IFs represent an essential epithelial keratin-like IF system necessary for the normal embryonic development of the hypodermis which is a prerequisite for elongation and locomotion of the worm (see also Introduction). The *second* IF group comprises proteins B2, D1, D2, and E1, which are exclusively expressed in the intestinal terminal web, and form probably another heteropolymeric system. Functionally this "B2/D/E1" IF system is involved in formation and/or regulation of the terminal web and possibly some other intestinal structures necessary for the normal function and development of one of the largest epithelial organs in *C. elegans*. Finally, the *third* IF group covers proteins C1 and C2. Both are the only IF proteins localized in the pharyngeal cellular junctions, while C2 is also clearly enriched in cellular junctions of the intestine (Karabinos et al., 2002a). In addition, both C proteins are also integrated into the terminal web possibly indicating their potential to serve as linkers connecting the terminal web IF cytoskeleton to CeAJ.

In conclusion, the work described here together with earlier results (Karabinos et al., 2001, 2003) has allowed us to describe the RNAi phenotype for 10 of the 11 *C. elegans* IF genes. A functional analysis of the last gene A4 should be possible in the future using transgenic animals expressing A4 dominant negative mutations. Surprisingly, nearly all RNAi phenotypes relate to the late embryo. Only A2 (early larval lethality) and C2 (adult phenotype) affect a later developmental stage. Five of the late embryonic lethal phenotypes (A1, A3, B1, C1, and D2) arise by single gene silencing. In contrast, in the mouse an embryonic lethal phenotype was not obtained in many different knockouts of IF genes. It was however observed when two genes encoding keratins of interior epithelia were knocked out simultaneously. Curiously, however, the defect involves fragility of the trophoblast giant cells and not the embryo proper (Hesse et al., 2000; Tamai et al., 2000) (see however also (Jaquemar et al., 2003)). Why is the *C. elegans* embryo so sensitive to loss of particular IF proteins? Part of the answer lies in the unique function of the hypodermis. Nematode hypodermal IFs are necessary for the strict coupling of hypodermal and muscle development and are also necessary for locomotion due to transmission of muscle force from the muscle to the cuticle

(Francis and Waterson, 1991; Hresko et al., 1994, 1999), i.e. for crucial functions of the *C. elegans* hypodermis. We previously speculated that the lack of cytoplasmic IFs in *Drosophila* reflects a cytoskeletal alteration in which bundles of microtubules can substitute for IFs (Karabinos et al., 2001). Given the lack of embryonic phenotypes in knockouts of various IF genes in the mouse one also wonders whether the actin microfilament system can at least in part substitute for IFs in the mammalian embryo.

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