

Type IV Collagen Is Detectable in Most, but Not All, Basement Membranes of *Caenorhabditis elegans* and Assembles on Tissues That Do Not Express It

Patricia L. Graham, Jeffrey J. Johnson, Shaoru Wang, Marion H. Sibley, Malini C. Gupta, and James M. Kramer

Northwestern University Medical School, Department of Cell and Molecular Biology, Chicago, Illinois 60611

Abstract. Type IV collagen in *Caenorhabditis elegans* is produced by two essential genes, *emb-9* and *let-2*, which encode $\alpha 1$ - and $\alpha 2$ -like chains, respectively. The distribution of EMB-9 and LET-2 chains has been characterized using chain-specific antisera. The chains colocalize, suggesting that they may function in a single heterotrimeric collagen molecule. Type IV collagen is detected in all basement membranes except those on the pseudocoelomic face of body wall muscle and on the regions of the hypodermis between body wall muscle quadrants, indicating that there are major structural differences between some basement membranes in *C. elegans*. Using lacZ/green fluorescent protein (GFP) reporter constructs, both type IV collagen genes were shown to be expressed in the same cells, primarily body wall muscles, and some somatic cells of the gonad. Al-

though the pharynx and intestine are covered with basement membranes that contain type IV collagen, these tissues do not express either type IV collagen gene. Using an epitope-tagged *emb-9* construct, we show that type IV collagen made in body wall muscle cells can assemble into the pharyngeal, intestinal, and gonadal basement membranes. Additionally, we show that expression of functional type IV collagen only in body wall muscle cells is sufficient for *C. elegans* to complete development and be partially fertile. Since type IV collagen secreted from muscle cells only assembles into some of the basement membranes that it has access to, there must be a mechanism regulating its assembly. We propose that interaction with a cell surface-associated molecule(s) is required to facilitate type IV collagen assembly.

BASEMENT membranes are thin sheets of specialized extracellular matrix that underlie or surround groups of cells, separating them from other cells and/or adjacent connective tissue. The major constituents of basement membranes are type IV collagen, laminin, nidogen (entactin), and heparan sulfate proteoglycan (perlecan) (59, 72). The functions and interactions of these molecules have primarily been studied in vertebrates using in vitro systems. These proteins, and presumably their functions, have been conserved throughout metazoan evolution. We are analyzing type IV collagen in vivo in the nematode *Caenorhabditis elegans* (26, 27) to further understand the assembly and functions of this basement membrane molecule.

The structure of type IV collagen has been conserved from nematodes to mammals (27, 29). The majority of the molecule is the central Gly-X-Y repeat domain, which folds into a triple-helical structure. There are ~ 20 sites at which the triple-helical structure is interrupted by amino acids in which glycine is not at every third position. At the amino terminus, there is a short non-(Gly-X-Y) domain containing four conserved cysteines, and at the carboxyl terminus, there is the globular NC1 domain that contains 12 conserved cysteines. In basement membranes, type IV collagen molecules form a complex polygonal network stabilized by intermolecular disulfide bonding (59). NC1 domains associate to form dimers, while amino terminal (7S) domains associate into tetramers. Lateral associations between type IV molecules are also involved in forming the network seen in basement membranes (70, 71).

Six type IV collagen genes have been identified in mammals, $\alpha 1$ – $\alpha 6$ (22, 29). The predominant form of type IV collagen in mammals is a heterotrimer of two $\alpha 1$ and one $\alpha 2$ (IV) chains. There are two genes that encode type IV collagen chains in *C. elegans*, *emb-9* and *let-2* (19, 20, 50).

Address all correspondence to James M. Kramer, Northwestern University Medical School, Department of Cell and Molecular Biology, 303 E. Chicago Ave., Chicago, IL 60611. Tel.: (312) 503-7644. Fax: (312) 503-2522 or 7912. E-mail: jkramer@nwu.edu

Marion H. Sibley's current address is New England Biolabs, Beverly, MA 01915.

EMB-9 is an $\alpha 1(\text{IV})$ -like chain and LET-2 is an $\alpha 2(\text{IV})$ -like chain. The six mammalian genes are organized as closely spaced pairs of one $\alpha 1$ -like and one $\alpha 2$ -like gene in head-to-head orientation (22, 29). In contrast, the *C. elegans* type IV collagen genes are located on different chromosomes. Two type IV collagen genes have been identified in sea urchin (9, 10), and one each in *Drosophila* (3, 4), and the parasitic nematodes *Brugia* (7) and *Ascaris* (46). There is no evidence for more than two type IV collagen genes in any invertebrate.

The diversity of type IV collagen is increased in *C. elegans* by alternative splicing of the $\alpha 2(\text{IV})$ chain gene *let-2* (50). One splice variant is predominant in embryos, while the other is predominant in larvae and adults. The alternative splicing does not change the length of the molecule but changes a small number of amino acids in the Gly-X-Y repeat domain. The same alternative splicing occurs in the $\alpha 2(\text{IV})$ chain of *Ascaris* (47). The importance of these splice variants is unknown, but they may increase the complexity of type IV collagen functions.

The predominant form of type IV collagen in mammals, $(\alpha 1)_2\alpha 2$, is abundant in most basement membranes (22, 24, 48). The four additional mammalian chains, $\alpha 3$ – $\alpha 6(\text{IV})$, have more restricted tissue distributions, being most abundant in the kidney (37, 43, 45, 68). Mutations in the human $\alpha 3$, $\alpha 4$, and $\alpha 5(\text{IV})$ collagen genes can cause Alport syndrome, a progressive glomerulonephritis variably associated with hearing loss and ocular lesions (2, 31, 41, 60). A similar nephrotic syndrome is seen in dogs with mutations in the $\alpha 5(\text{IV})$ collagen gene (73) and in mice with a knockout of the $\alpha 3(\text{IV})$ collagen gene (8, 38). The primarily renal focus seen in Alport syndrome is consistent with the localization of the $\alpha 3$ – $\alpha 5(\text{IV})$ chains primarily in kidney. Mutations have not been identified in the mammalian $\alpha 1$ or $\alpha 2(\text{IV})$ collagen genes.

Mutations have been characterized in the *C. elegans* $\alpha 1$ and $\alpha 2(\text{IV})$ collagen genes, *emb-9* and *let-2* (20, 20a, 51). Mutations in either of the two genes cause similar defects. Most of the mutations are substitutions for glycines in the Gly-X-Y repeat domain and cause temperature-sensitive phenotypes. At nonpermissive temperature (25°C), most of these mutations cause arrest at the twofold stage of embryonic development. At intermediate temperature (20°C), they generally cause larval arrest or adult sterility. At the permissive temperature (15°C), most of the mutations allow animals to develop and reproduce. Thus, normal EMB-9 and LET-2 are required for both viability and fertility. The evidence that *emb-9* and *let-2* are the only type IV collagen genes in *C. elegans* and their embryonic lethal phenotypes suggest that their products may be widely distributed, analogously to the mammalian $\alpha 1$ and $\alpha 2$ chains.

Here we show that the EMB-9 and LET-2 type IV collagen chains are colocalized and are found in most, but not all, basement membranes in *C. elegans*. We show that the *emb-9* and *let-2* genes are expressed in mesoderm, primarily body wall muscle and somatic gonadal cells. We demonstrate that EMB-9 produced in body wall muscles can assemble into the basement membranes that surround other tissues and that body wall muscle expression is sufficient for *C. elegans* development. Our results indicate that a mechanism must exist to control the sites of assembly of type IV collagen in basement membranes.

Materials and Methods

C. elegans Strains

Animals were maintained as described by Brenner (6). The *C. elegans* variety Bristol isolate N2 is designated as wild-type. The mutant strain used in this study, *unc-36(e251) emb-9(g23cg45)/qCJ[unc-36(+)/emb-9(+)]*, was generated by reverting the temperature-sensitive dominant lethality of *emb-9(g23)* (20a). The *cg45* mutation converts Gln1388 of EMB-9 to a termination codon.

Preparation of Type IV Collagen Antisera

cDNA fragments encoding the entire NC1 domains of EMB-9 and LET-2 were generated by reverse transcriptase-PCR amplification and cloned into pGEX fusion protein vectors (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Fusion proteins were isolated in inclusion bodies after overexpression in *Escherichia coli*. The inclusion bodies were solubilized in SDS/ β -mercaptoethanol sample buffer (30) and electrophoresed on SDS-polyacrylamide gels, and the fusion protein bands were excised from the gels and used to immunize rabbits. Synthetic peptides derived from the NC1 domain of EMB-9 (CVDQDKQFRKPMQ) or from exon 9 of LET-2 (CREFTGSGSIVGPR) were coupled to BSA using *m*-maleinido-benzoyl-*N*-hydroxysuccinimide ester (21).

Rabbit antisera against fusion protein and peptide antigens were generated commercially (Hazelton Research Products, Inc., Denver, PA). Sera against synthetic peptides were affinity purified using Actigel (Serogene Bioseparations, Arcadia, CA) columns coupled with the same peptide. Fusion protein antisera were first affinity purified on columns coupled with the fusion protein to which they were generated. Cross-reacting antibodies were then removed by passing the affinity-purified anti-EMB-9 sera over a column containing the LET-2 fusion protein, and vice versa. Purified antisera were stored at -20°C in PBS with 50% glycerol, 1% BSA.

Western Blots

C. elegans extracts were prepared by washing mixed-stage populations extensively with large volumes of distilled water (dH_2O).¹ After pelleting, they were mixed immediately in an equal volume of $2\times$ Laemmli sample buffer (30), containing 5% freshly added β -mercaptoethanol, and boiled for 10 min. Extracts were then incubated for 12–24 h at room temperature, boiled 10 min, and stored at -20°C .

Western blots were performed by standard procedures (21). *C. elegans* protein extracts were electrophoresed on either 7.5 or 4% polyacrylamide gels and transferred to nitrocellulose using 25 mM Tris, 192 mM glycine, 0.1% SDS, 10% methanol. Nitrocellulose filters were stained with Ponceau S to determine the position of molecular mass markers and then blocked for at least 1 h in BB (BB: 10% dry milk, 0.3% Tween 20 in PBS). Filters were incubated with anti-type IV collagen antibodies diluted 1:500–1:1,000 in BB for 1 h at room temperature and washed with 0.3% Tween 20 in PBS. They were then incubated with alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma Chemical Co., St. Louis, MO) diluted 1:1,000 in BB, washed, and developed with BCIP/NPT (21).

For competition experiments, anti-type IV collagen antibodies were preincubated for 1 h at room temperature with 200–400 $\mu\text{g}/\text{ml}$ of the fusion protein or 100 $\mu\text{g}/\text{ml}$ of the peptide used as the original antigen. The antibody/antigen mixture was then used to stain blots as described above, or to stain animals as described below.

Antibody Staining of *C. elegans*

Animals were prepared for immunofluorescence analysis by one of three methods. In the first, eggs were collected from alkaline hypochlorite-treated animals (55), rinsed twice with dH_2O , fixed with 3% paraformaldehyde in PBS for 15 min at room temperature, rinsed twice with dH_2O , and stored at -80°C in 100% methanol for 30 min to 7 d. Alternatively, animals in dH_2O were pipetted onto slides subbed with 0.2% gelatin, 0.1% polylysine, 0.01% chrome alum, covered with a coverslip, and frozen on dry ice. The coverslip was cracked off with a razor blade, and the slide was immersed in -20°C methanol for 5 min, followed by -20°C acetone for 3 min, and air dried. Lastly, larvae and adults were prepared as described by Finney and Ruvkun (11).

1. Abbreviations used in this paper: dH_2O , distilled water; GFP, green fluorescent protein; HA, hemagglutinin; NDS, normal donkey serum.

After fixation, animals were rehydrated and either blocked immediately for 1 h in PBS containing 0.1% Triton X-100 (PBS-T) and 10% normal donkey serum (NDS), or treated with 6 M urea, 0.1 M glycine, pH 3.5, for 15 min (69) and rinsed 3× with PBS before blocking. Samples were incubated with antibodies against type IV collagen (EMB-9 or LET-2), myosin heavy chain B (UNC-54), or mouse anti-hemagglutinin (HA) monoclonal antibody (Boehringer-Mannheim Corp., Indianapolis, IN) in PBS-T-NDS overnight at 4°C for 1 h at 37°C, washed with PBS-T, incubated 1 h at 37°C with FITC donkey anti-rabbit and LRSC donkey anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA) in PBS-T-NDS, washed with PBS-T containing 1 μg/ml diamidinophenolindole, followed by PBS alone.

To prepare frozen sections, animals were fixed with 3% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.0, for 20 min at room temperature. After washing in water, they were resuspended in 30% sucrose in PBS at 4°C. After the animals sank to the bottom of the tube, the overlying sucrose solution was removed and an equal volume of OCT compound (Miles, Inc., Elkhart, IN) was added. Frozen sections were collected on polylysine-coated slides and dried for 30 min before being blocked and stained as described above.

Construction and Analysis of Type IV Collagen Expression Reporters

Reporter constructs for *emb-9* were generated from the plasmid pJJ359, which contains the complete *emb-9* gene flanked by 4.3 kb of 5' upstream and 0.5 kb of 3' sequence (Fig. 1 A). pJJ359 is capable of transgenic rescue of *emb-9* mutant animals. To generate the *emb-9::lacZ* reporter construct pJJ444, the 10-kb XmaI-SaII fragment from pJJ359 was inserted into XmaI-XhoI cut pPD34.110. pPD34.110 contains the *E. coli lacZ* gene with a membrane stop transfer sequence and 3' untranslated sequences from *unc-54* (13.). An *emb-9::green fluorescent protein (GFP)* reporter, pJJ446, was made by removing the *lacZ* region of pJJ444 by KpnI-SpeI digestion and inserting the KpnI-SpeI GFP-containing fragment from pPD95.75 (provided by A. Fire, S. Xu, J. Ahnn, and G. Seydoux).

Another *emb-9::lacZ* reporter construct, pJJ318, was made by inserting

a HindIII-PvuII (−1672 to −18 relative to *emb-9* start codon) fragment from the lambda phage clone CH#2 into HindIII-SmaI-digested pPD21.28. CH#2 contains the complete *emb-9* gene and is capable of transgenic rescue of *emb-9* mutant animals (20). pPD21.28 contains the *E. coli lacZ* gene with a nuclear localization signal and 3' untranslated sequences from *unc-54* (13).

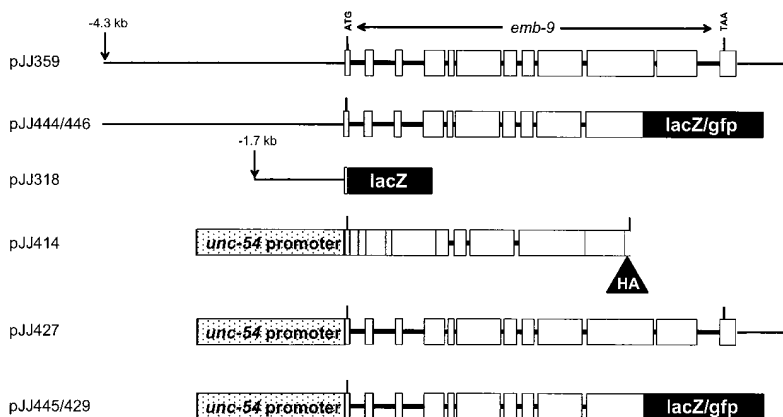
Two *let-2* reporter constructs were generated from the plasmid pJJ353, which contains the complete *let-2* gene with 2.2 kb of 5' upstream and 430 bp of 3' untranslated sequence (Fig. 1 B). pJJ353 is capable of transgenic rescue of *let-2* mutant animals (50). pJJ360 was made by ligating the 6-kb SphI-EcoRV fragment from pJJ353 into SphI-SmaI-digested pPD34.110. pJJ361 was made by ligating the 4-kb SmaI-ApaI fragment from pPD34.110 with the 11-kb EcoRV-ApaI fragment from pJJ353. pPD34.110 contains the *E. coli lacZ* gene with a membrane stop transfer sequence and 3' untranslated sequences from *unc-54* (13). An additional *let-2* reporter, pJJ325, was generated by cloning the 3.2-kb AvaI fragment from cosmid C12F7 (−3.2 to +20 kb relative to *let-2* initiation codon) into SmaI-digested pPD22.04 (13).

DNAs were injected into hermaphrodite gonads as described (36) using pRF4[*rol-6(su1006)*] (28) as a transformation marker. The *emb-9* reporter construct pJJ318 was integrated into the genome by γ-irradiation and selection of a line that segregates 100% roller progeny. β-galactosidase activity was visualized by staining as described (12), after fixation for 15 min with 3% paraformaldehyde in PBS and washing with PBS.

Construction and Analysis of Epitope-tagged Type IV Collagen

The pJJ400 construct was made by cloning the 3.6-kb HindIII-XbaI fragment from pJJ359 (Fig. 1 A) into the pCRII vector (InVitrogen, San Diego, CA), which is useful because it has no AatII sites. PCR was performed on first strand cDNA derived from mixed-stage *C. elegans* RNA using primers *emb9ectA* (−44 to −9, relative to translation start) and *emb9ectB2* (+3268 to +3250). *Emb9ectA* has a single nucleotide change, T-38G, relative to wild-type to create a HindIII site. *Emb9ectB2* has a synthetic HindIII site appended. PCR products were HindIII digested and

A *emb-9* constructs



B *let-2* constructs

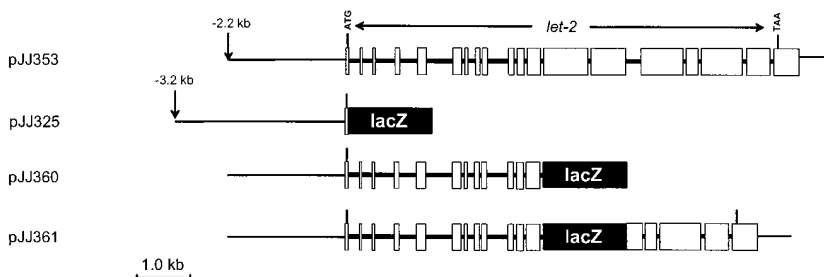


Figure 1. Diagrams of *emb-9* and *let-2* expression constructs. Constructs involving *emb-9* were derived from the genomic clone pJJ359 (A). Constructs involving *let-2* were derived from the genomic clone pJJ353, or cosmid C12F7 (B). Exons are indicated as open boxes, introns as thick lines, and flanking sequences as thin lines. The *unc-54* promoter/enhancer is stippled, and the HA epitope tag and reporter sequences are solid. The *emb-9* and *let-2* genomic sequences are drawn to scale, but exogenous sequences are not.

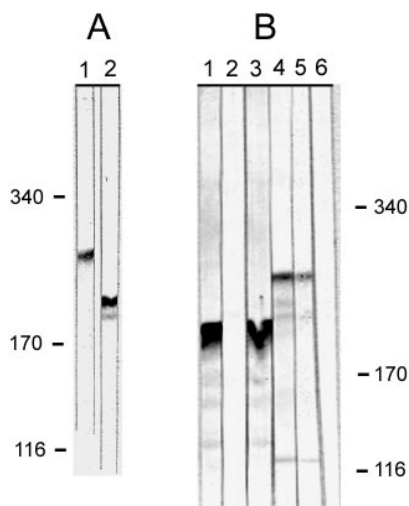


Figure 2. Antibodies against the $\alpha 1$ and $\alpha 2(\text{IV})$ collagen chains, EMB-9 and LET-2, react specifically to their respective antigens. Western blots of *C. elegans* extract were probed with type IV collagen antisera. (A) Strips cut from a blot of *C. elegans* extract were reacted with the anti-EMB-9 peptide antiserum 1910 (lane 1) or the anti-LET-2 peptide antiserum 68 (lane 2). (B) *C. elegans* extract was reacted with fusion protein-generated antiserum NW153 against LET-2 (lanes 1–3) or antiserum NW155 against EMB-9 (lanes 4–6). Before reacting with the blot, the antibodies were incubated with no competitor (lanes 1 and 4), LET-2 fusion protein (lanes 2 and 5), or EMB-9 fusion protein (lanes 3 and 6). Antibodies incubated with the antigen to which they were made no longer react to the worm extract (lanes 2 and 6), while those incubated with the other fusion protein still react to the appropriate size band (lanes 3 and 5).

cloned into HindIII-digested pJJ400 to generate pJJ401. The 1.6-kb AatII fragment was removed from pJJ401 and replaced with the 1.6-kb AatII fragment from pJJ359 to generate pJJ402. PCR was performed on first strand cDNA derived from mixed-stage *C. elegans* RNA using primers emb9ectD (+5725 to +5750) and embtwaHA, which is the complement of +7217 to +7231 with two HA epitope tags (67), a termination codon, and an Xba site appended. PCR products were SaII-XbaI digested and cloned into SaII-XbaI-digested pJJ402 to generate pJJ404. The insert in pJJ404 was isolated after NsiI-ApaI digestion and ligated into NheI-KpnI-digested pPD30.38, after both fragments had been blunt ended with T4 DNA polymerase. pPD30.38 contains the *unc-54* body wall muscle myosin promoter and enhancer (35). The resulting clone, pJJ414 (Fig. 1 A), has the *unc-54* promoter and enhancer, the *emb-9* gene with introns 1–6, 10, and 11 removed and two copies of HA inserted immediately preceding the termination codon, and 3' untranslated sequences from pPD30.38.

Transgenic Rescue of *emb-9*(null) by Expression in Muscle Cells

Plasmid pJJ427 was constructed by ligating the 3.5-kb ApaI-KpnI fragment from pPD30.38 with the 7.8-kb PvuI-PstI fragment from pJJ359 after blunt ending. This construct has the wild-type *emb-9* gene (–15 to +7750) under control of the *unc-54* promoter and enhancer. pJJ427 was used to generate transgenic strains in *unc-36(e251) emb-9(g23cg45)/qC1* animals. The constructs pJJ445 and pJJ429 were generated by ligating the *emb-9* gene and reporter containing NsiI-SpeI fragments from pJJ444 or pJJ446 with the *unc-54* promoter containing NsiI-XbaI fragment from pJJ427.

Results

Characterization of Anti-type IV Collagen Antisera

We have used four antisera to examine the distribution of

type IV collagen chains in *C. elegans*. Two antisera were generated against fusion proteins containing the NC1 domains of EMB-9 (NW155) or LET-2 (NW153), one against a peptide from the NC1 domain of EMB-9 (NW1910), and one against a peptide derived from an interruption of the Gly-X-Y domain of LET-2 (NW68). Each of the sera was affinity purified using the original antigen. In addition, the fusion protein sera were cross adsorbed on columns carrying the fusion protein from the other type IV collagen chain. The purified anti-EMB-9 antisera do not cross-react with the LET-2 fusion protein, and the purified anti-LET-2 antisera do not cross-react with the EMB-9 fusion protein (data not shown).

On Western blots of total *C. elegans* extracts, both EMB-9 antisera react with a major band of ~ 240 kD, and both LET-2 antisera react with a major band of ~ 210 kD (Fig. 2). The predicted molecular masses of EMB-9 and LET-2, after cleavage of signal peptides, are 168 and 165 kD, respectively. Their higher apparent molecular masses are at least partly due to the abnormally slow migration of collagens relative to globular protein molecular mass standards on SDS-polyacrylamide gels (17). The apparent molecular masses of the reacting bands differ depending on the percentage acrylamide in the gels, another characteristic of collagens. The fact that the EMB-9 and LET-2 antisera specifically react with proteins of different mobilities indicates that they are specific for the chains against which they were made. Activity on Western blots is eliminated by competition with the original antigen, but not by competition with antigen from the other type IV collagen chain (Fig. 2 B). Similar results were obtained in competition experiments on embryos prepared for immunofluorescence. The specificity of the antisera is further supported by the fact that the anti-EMB-9 antisera do not react with embryos homozygous for either a stop codon or an internal deletion of *emb-9* (20a).

Distribution of Type IV Collagen

Ultrastructural analyses of *C. elegans* (1, 64–66) have identified basement membranes on the pseudocoelomic face of the hypodermis and surrounding the body wall muscles, pharynx, intestine, and gonad (Fig. 3). We have determined the distributions of the $\alpha 1$ and $\alpha 2(\text{IV})$ collagen chains EMB-9 and LET-2 in whole mount animals using specific anti-type IV collagen antisera. All four antisera generate essentially identical immunofluorescence patterns, so below we refer to type IV collagen localization rather than to the separate chains. The colocalization of EMB-9 and LET-2 chains further supports the specificity of the antisera and suggests that EMB-9 and LET-2 may assemble into the same heterotrimeric collagen molecule.

At 20°C, *C. elegans* embryogenesis takes ~ 800 min from first division to hatching (55). Most cell divisions are completed during the first 350 min, after which the embryo begins elongation from an ovoid ball into a tube approximately four times the length of the egg. We refer to the stages of morphogenesis, from the start of elongation to hatching, by the length of the embryo relative to the egg shell, progressively as lima, comma, 1 1/2-fold, twofold, threefold, and fourfold.

Type IV collagen is first detectable in body wall muscle

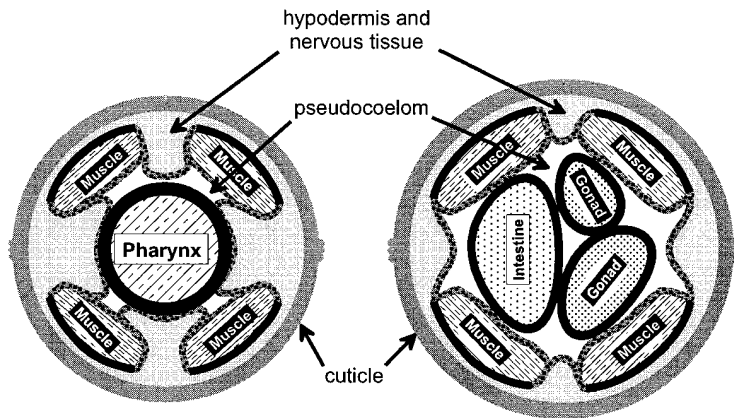


Figure 3. Schematic cross sections of *C. elegans* through the head (left) and midbody (right) showing the locations of basement membranes. Basement membranes that stain with anti-EMB-9 and anti-LET-2 antisera are shown as thick black lines; those that do not stain are shown as thick stippled lines. Tissues that express the *emb-9* and *let-2* genes are indicated with white text on a black background; those that do not are shown as black text on a white background. Note that only a subset of somatic gonadal cells express *emb-9* and *let-2*.

Basement membranes that:

- stain for type IV collagen
- ▨ do not stain for type IV collagen

Tissues that express type IV collagen:

- Gonad**
- Muscle**

Tissues that do not express type IV collagen:

- Intestine**
- Pharynx**
- Hypodermis**

cells at the onset of morphogenesis, just preceding the lima stage (data not shown). At this early stage, the type IV collagen stain appears to be completely intracellular. By the 1 1/2-fold stage, strong type IV collagen stain is seen in body wall muscle cells and at the interface between them and the hypodermis (Fig. 4, A and B). Weak stain is seen surrounding the pharyngeal and intestinal primordia beginning at the comma stage, and this stain becomes intense by the 1 3/4-fold stage (Fig. 4, C and D). Intracellular stain is also seen in the head mesodermal cell and its lineal homologue MS.pppaaa by this stage (Fig. 4, E and F).

From about the comma stage on, stain for type IV collagen accumulates in four stripes at the interfaces between the hypodermis and the four body wall muscle quadrants (Fig. 4, G–J). There is no stain detectable in the hypodermal basement membrane that is located between the muscle quadrants. There is also no detectable stain on the pseudocoelomic face of body wall muscle cells. This same staining pattern is seen with all four type IV antisera and is not altered after treatment with acid urea to unmask potentially hidden epitopes (69).

After the twofold stage, six cells in the nerve ring frequently exhibit stain for type IV collagen (Fig. 4 K). Based on their positions, these cells appear to be the GLR cells, which are considered to have glial-like characteristics (66). The GLR cells have broad thin processes that separate the muscle arms that project under the nerve ring from the pharynx. At the time of hatching, strong type IV collagen stain is seen at the interface between the body wall muscle cells and the hypodermis and surrounding the pharynx, intestine, and primordial gonad (Fig. 4 L).

In larval and adult animals, the distribution of type IV collagen generally remains the same as that seen in late embryos; underlying the body wall muscles and surrounding the pharynx, intestine, and gonad (Fig. 5 C). As in embryos, type IV collagen is not detectable in the hypodermal basement membranes between body wall muscle quadrants or on the pseudocoelomic face of body wall muscles (Fig. 5, A and B). In larvae and adults, coelomocytes can

be seen to stain with the type IV antisera (Fig. 5 D). The basement membrane surrounding the gonad stains for type IV collagen throughout larval development and in adults. However, in many L4 and adult animals the gonadal stain is not uniform, being more intense around the spermatheca (Fig. 5 E) and around the distal ends of the gonad arms. Although the basement membranes surrounding the pharynx and intestine stain with the type IV antisera, intracellular stain in these tissues was never seen in embryos, larvae, or adults. A schematic summary of the observed type IV collagen staining pattern is shown in Fig. 3.

Type IV Collagen Gene Expression

The sites of *emb-9* and *let-2* expression have been determined using lacZ and GFP reporter constructs in transgenic animals. For both genes, the reporter constructs were derived from clones that are capable of rescuing mutant animals when present in transgenic arrays. For *emb-9*, constructs in which lacZ or GFP are inserted into exon 10 and that have 4.3 kb of 5' upstream sequences, pJJ444/446 (Fig. 1 A), show strong activity in the cells that exhibit intracellular stain with anti-EMB-9 antisera. A construct with 1.7 kb of 5' upstream sequences in a transcriptional fusion with lacZ, pJJ318, has also been analyzed, both as an extrachromosomal array and integrated into the genome. This construct has an expression pattern very similar to pJJ444 but often shows activity in two to four of the most posterior intestinal cells in early larvae and has lower activity in body wall muscles of late larvae and adults.

For *let-2*, 3.2 kb of 5' upstream sequences, which is more than is required for transgenic rescue of mutant animals, shows very little activity when used in a transcriptional fusion with lacZ, pJJ325 (Fig. 1 B). However, insertion of lacZ into exon 13 of *let-2* generates a translational fusion, pJJ360, that shows strong activity in the same cells that stain intracellularly with type IV collagen antisera. The presence of further 3' exons and flanking sequences, pJJ361, have no effect on the observed expression pattern. The *let-2*

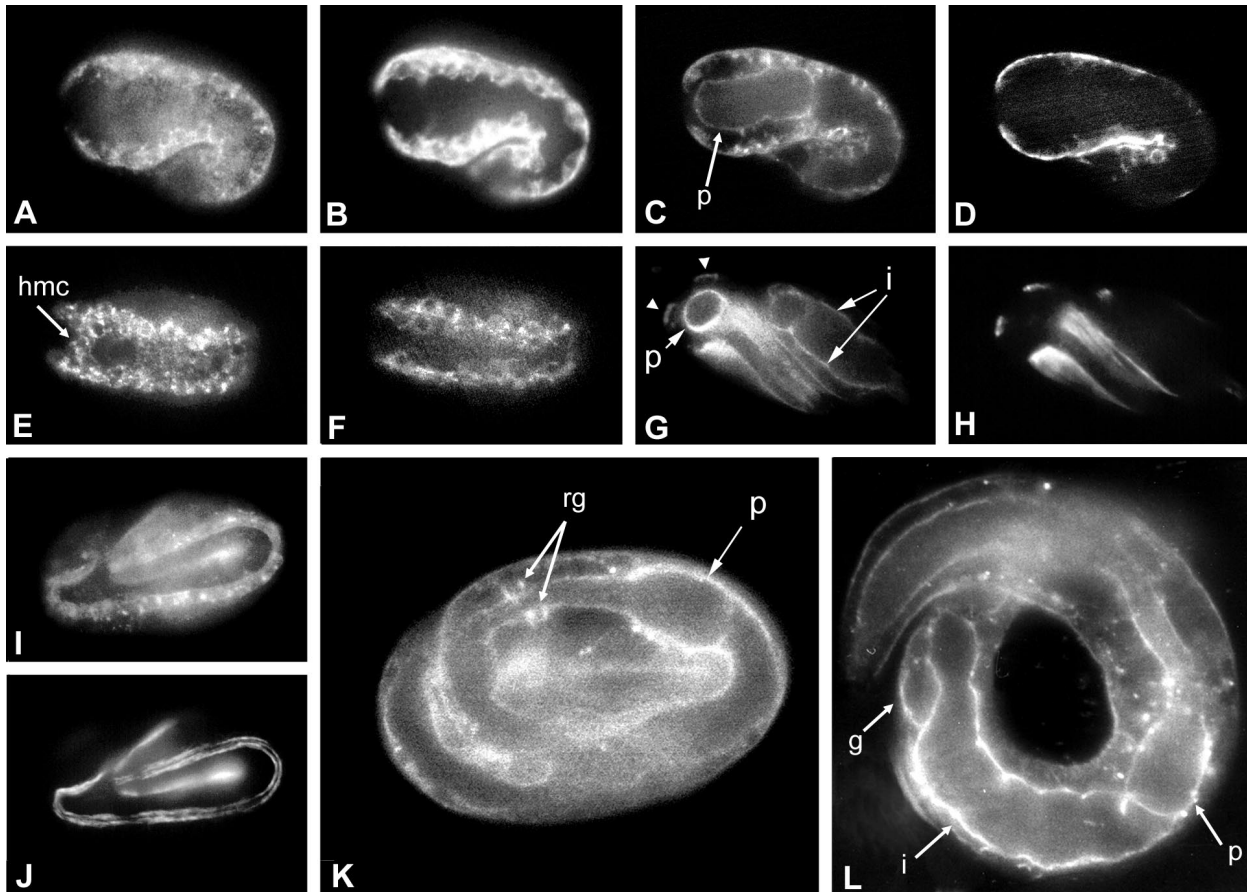


Figure 4. Type IV collagen distribution in wild-type *C. elegans* embryos. Embryos were stained with anti-LET-2 antiserum 68 (A, E, G, and K) or anti-EMB-9 antiserum 1910 (C, I, and L). The same staining patterns are seen with all anti-EMB-9 and anti-LET-2 antisera. The same embryos were stained with anti-UNC-54 myosin monoclonal antibody to identify body wall muscle cells (B, D, F, H, and J). (A) A 1 1/2-fold embryo shows type IV collagen accumulation in body wall muscle cells. (B) Anti-UNC-54 staining of the same embryo in A. (C) A 1 3/4-fold embryo shows type IV collagen in body wall muscle cells and covering the pharynx primordium (p). (D) Anti-UNC-54 staining of the same embryo in C. (E) A 1 1/2-fold embryo showing type IV collagen in body wall muscle cells, and the head mesodermal cell (hmc) and its lineal homologue. (F) Anti-UNC-54 staining of the same embryo in E. Note that the hmc and its homologue do not stain for UNC-54. (G) A twofold embryo showing type IV collagen staining under the body wall muscle quadrants (two of the four are indicated with arrowheads), but not between them. Stain around the pharynx (p) appears as a ring in the head in this optical cross-section. Stain around the intestine (i) is seen in the posterior of the embryo. (H) Anti-UNC-54 staining of the same embryo in G. (I) Surface focal plane of a threefold embryo shows type IV collagen as a stripe overlying a body wall muscle quadrant. (J) Anti-UNC-54 staining of the same embryo in I. (K) Type IV collagen staining of two ring ganglion cells (rg), believed to be GLR cells, in a fourfold embryo. The posterior bulb of the pharynx (p) is indicated. (L) An embryo at hatching showing type IV collagen stain of basement membranes covering the pharynx (p), intestine (i), and gonad primordium (g).

expression reporters, pJJ360/361, show the same spatial and temporal expression patterns as the *emb-9* pJJ444/446 reporter constructs.

Weak β -galactosidase and GFP activity can first be detected in body wall muscle cells of lima stage embryos (data not shown). In embryos of all later stages, β -galactosidase and GFP activity is strong in body wall muscle cells (Fig. 6, A and B), and no activity is seen in pharyngeal, intestinal, or hypodermal cells. In situ hybridization of *C. elegans* embryos with a probe derived from *emb-9* also shows localization in the body wall muscles (56). In L1 larvae (Fig. 6 D), body wall muscle cells continue to show activity, and activity is also seen in the ring ganglion (presumptive GLR cells), head mesodermal cell, coelomocytes, and intestinal and anal muscles. Beginning at the L2 larval stage, the distal tip cells of the developing gonad show strong activity

(Fig. 6 C). In L3–L4 animals, the level of activity in the distal tip cells often appears to be greater than that seen in the body wall muscles (Fig. 6 E). In L4 animals, spermathecal cells begin to show β -galactosidase activity, and this activity can still be seen in adults (Fig. 6 F). In adult animals, type IV collagen reporter activity is seen in vulval and uterine muscles (Fig. 6 F), body wall muscles (Fig. 6 G), as well as distal tip cells (Fig. 6 H).

The *emb-9* and *let-2* reporters showed activity in the same cells that stained with the anti-EMB-9 and -LET-2 antisera, with the exception of some accessory muscles. Although the intestinal, anal, vulval, and uterine muscles showed activity with type IV expression reporters, intracellular staining of these cells with anti-type IV collagen antisera was not apparent. It is possible that antibody staining of these cells was weak and/or obscured by the in-

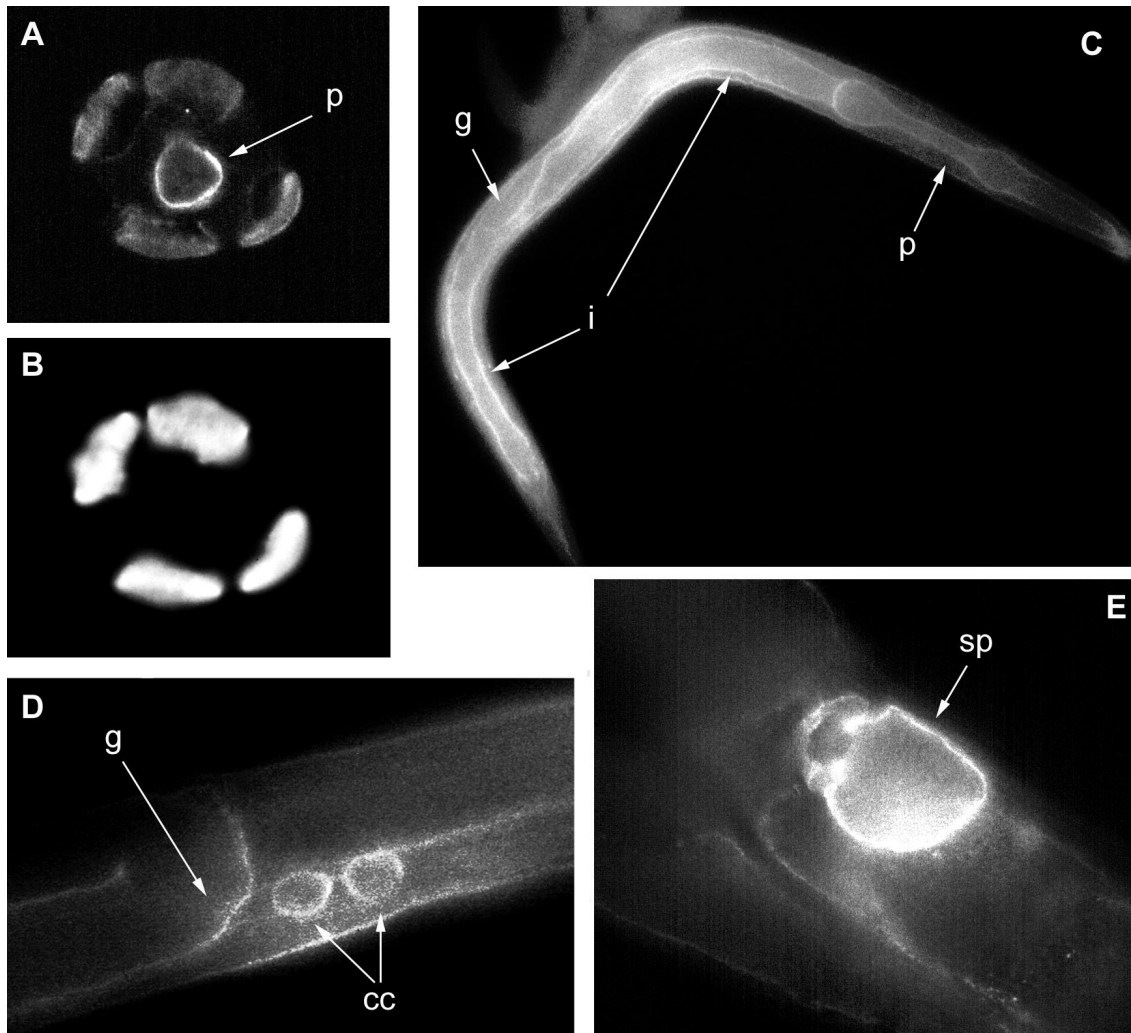


Figure 5. Type IV collagen distribution in wild-type *C. elegans* larvae and adults. Anti-LET-2 (A) and anti-UNC-54 myosin (B) stain of a frozen section through the head of an adult animal. The Type IV stain is associated with the pharynx (p) and four body wall muscle quadrants. There is not type IV collagen stain between muscle quadrants. Type IV collagen stain is localized near the myosin stain, which is closely apposed to the hypodermal face of body wall muscle cells. (C) Anti-EMB-9 stain of an L2 larva showing localization to basement membranes surrounding the pharynx (p), intestine (i), and gonad (g). (D) Anti-EMB-9 stain of an L4 larva showing reaction with the gonad basement membrane (g) and with two coelomocytes (cc). (E) Anti-LET-2 stain of an adult showing intense stain surrounding the spermatheca (sp).

tense stain of adjacent intestinal, gonadal, and body wall muscle basement membranes.

It is notable that no *emb-9* or *let-2* reporter activity was seen in pharyngeal, intestinal, or hypodermal cells of embryos, larvae, or adults. This result is consistent with the fact that anti-type IV collagen antisera did not show intracellular stain in these tissues. However, it is surprising because the pharynx and intestine are covered by basement membranes that do stain with the anti-type IV collagen antisera. Although the pharyngeal and intestinal basement membranes contain EMB-9 and LET-2, the genes encoding them are not expressed in these tissues.

Type IV Collagen Expressed in Muscle Cells Can Assemble on Other Tissues

The strong expression of *emb-9* and *let-2* in body wall muscle cells suggests that they could be the source of the type

IV collagen found in the pharyngeal and intestinal basement membranes. To determine if type IV collagen produced in body wall muscle cells can assemble on other tissues, we constructed an epitope-tagged *emb-9* gene that is transcriptionally controlled by the body wall muscle-specific *unc-54* promoter and enhancer, pJJ414 (Fig. 1 A). The *unc-54* gene encodes the body wall muscle myosin heavy chain MHC-B (33), and the specificity of its promoter has been well documented (44). The epitope tag consists of two tandem copies of the nine-amino acid HA peptide (67). Transgenic lines carrying the construct were generated in the wild-type N2 background and stained with anti-HA and -type IV collagen antibodies.

In lima through 1 1/2-fold embryos, the anti-HA stain is seen in body wall muscle cells (Fig. 7 A), coincident with stain for the LET-2 chain (Fig. 7 D). In later embryos, when wild-type type IV collagen is localized largely in basement membranes (Fig. 7 E), the HA-tagged mole-

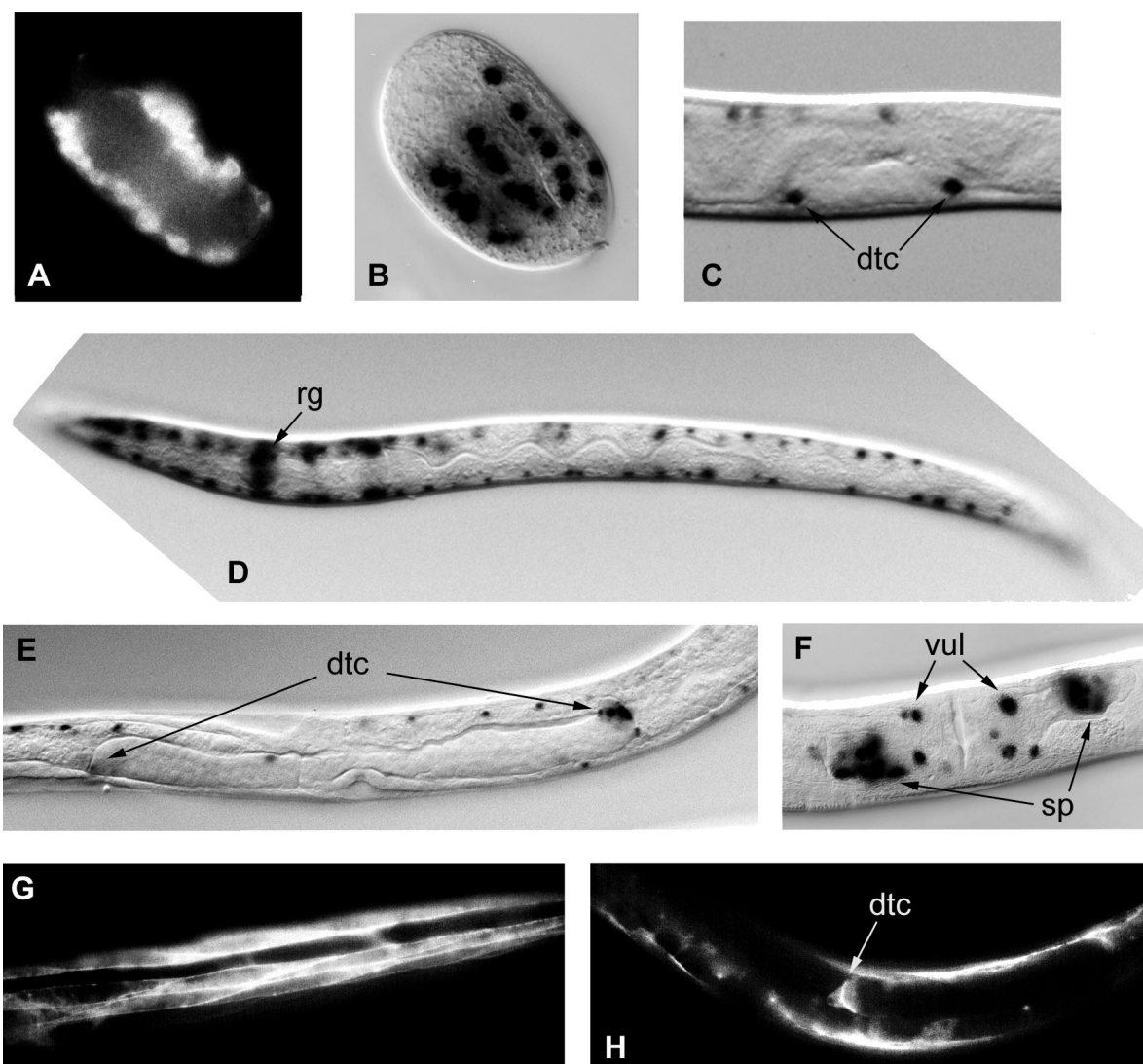


Figure 6. Expression of type IV collagen genes in *C. elegans*. Expression reporters for *emb-9* and *let-2* show activity in the same cells and at the same times. (A) A 1 1/2-fold embryo showing GFP activity from the *emb-9* reporter pJJ446 in body wall muscle cells. (B) A two-fold embryo showing β-galactosidase activity from pJJ318 in body wall muscle cells. (C) An L2 larva showing β-galactosidase activity from pJJ318 in the distal tip cells (*dtc*) of the gonad. (D) An L1 larva showing β-galactosidase activity from pJJ444 in body wall muscle cells and ring ganglion cells (*rg*). (E) A late L3 larva showing β-galactosidase activity from pJJ444 in the distal tip cells (*dtc*) of the gonad. (F) A young adult animal showing β-galactosidase activity from pJJ318 in spermathecal cells (*sp*) and vulval muscles (*vul*). (G) A young adult animal showing GFP activity from pJJ446 in body wall muscle cells. (H) An adult animal showing GFP activity from pJJ446 in a distal tip cell (*dtc*) of the gonad.

cules are mostly still intracellular (Fig. 7 B). This intracellular retention appears similar to that seen in animals with mutations in either of the type IV collagen genes (20a). In very late embryos that are close to hatching, weak HA staining is detectable on the surface of the pharynx and intestine (Fig. 7, C and F). Thus, the epitope-tagged EMB-9 chain is expressed in body wall muscle as expected; however, the presence of the HA epitope tag at the carboxyl end of EMB-9 appears to interfere with its proper assembly and/or secretion.

The inhibition of HA-tagged EMB-9 secretion is not complete, however, since in larvae and adults carrying pJJ414, strong staining with anti-HA antibodies is seen covering the pharynx and intestine (Fig. 7, G and H). This staining is coincident with that seen with anti-type IV col-

lagen antisera (Fig. 7, I and J). Staining with HA antibodies beneath body wall muscles and over the gonad can also be detected. Animals in the same preparation that do not carry the transgenic array show no staining, demonstrating the specificity of the HA antibody (Fig. 7, G–J). These results show that type IV collagen produced in body wall muscle cells can assemble into the basement membranes covering other tissue.

Expression of EMB-9 in Body Wall Muscle Can Rescue *emb-9* Mutant Animals

As a test of how much type IV collagen function can be provided by expression in body wall muscle cells, we asked how far animals could develop if they produced functional

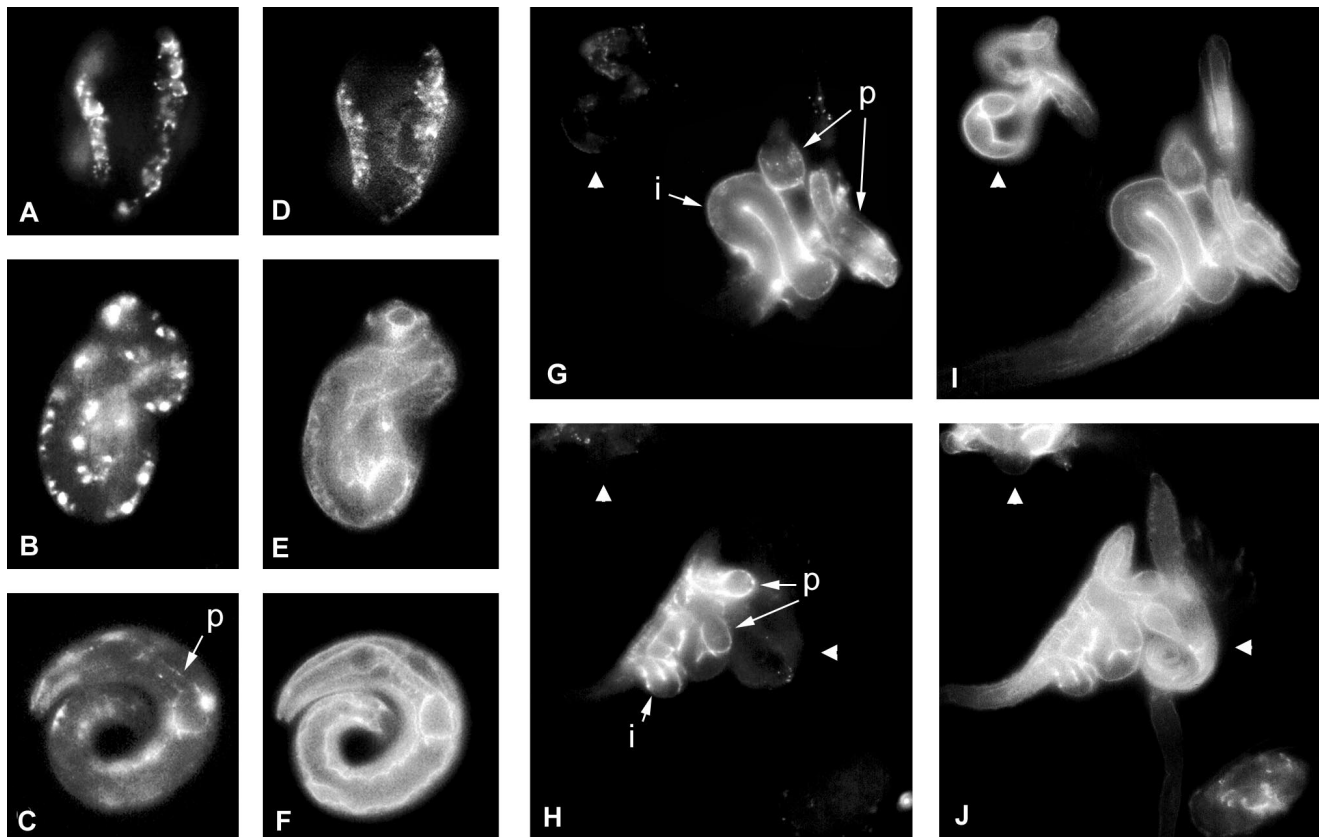


Figure 7. Distribution of HA-tagged EMB-9 expressed from the *unc-54* body wall muscle myosin promoter construct pJJ414. Animals were stained with anti-HA monoclonal antibody (A–C, G, and H), and with anti-LET-2 antiserum (D–F, I, and J). (A) In a 1 1/2-fold embryo, anti-HA staining colocalizes in body wall muscle cells with LET-2 (D). (B) In a threefold embryo, the HA-tagged EMB-9 is retained within the body wall muscle cells, while LET-2 is seen in basement membranes (E). (C) In a fourfold embryo, a small amount of HA-tagged EMB-9 can be seen in the pharyngeal basement membrane, while LET-2 staining of basement membranes is intense (F). In L2–L3 larvae, HA-tagged EMB-9 is seen in pharyngeal (p) and intestinal (i) basement membranes (G and H) and can also be detected under body wall muscle quadrants and around the gonad. Animals that do not carry the transgenic array, and do not stain with anti-HA antibody, are indicated with arrowheads. Staining of the same animals with anti-LET-2 (I and J) shows that the HA-tagged EMB-9 colocalizes with LET-2.

type IV collagen only in body wall muscle cells. The body wall muscle-specific *unc-54* promoter and enhancer were placed immediately upstream of the initiation codon of the complete wild-type *emb-9* gene to create pJJ414 (Fig. 1 A). This construct was used to generate transgenic arrays in animals carrying an *emb-9* nonsense mutation linked to an Unc marker maintained over a balancer chromosome, *unc-36* (*e251*) *emb-9*(*g23cg45*)/*qC1*. Normally, this strain segregates no Unc progeny because of the strict embryonic lethality of the *emb-9* nonsense mutation.

We expected that, at best, sterile adults could develop from *emb-9*(*g23cg45*) animals carrying pJJ427, since the normal gonadal type IV collagen expression would be absent. To our surprise, viable and fertile transgenic Unc animals were generated by injecting the *unc-54:emb-9* construct. We have established several lines that are homozygous *unc-36*(*e251*) *emb-9*(*g23cg45*) and are rescued by arrays carrying the pJJ427 construct. The rescued animals grow slowly; many arrest during larval development, and those that reach adulthood have small brood sizes. However, the strains can be maintained. These results indicate that expression of functional type IV collagen only in body wall

muscles is sufficient for *C. elegans* to develop to adulthood and to reproduce.

It is possible that the presence of the complete *emb-9* coding region in the pJJ427 construct could alter the expression pattern of the *unc-54* promoter and enhancer. To test this possibility, GFP was inserted into exon 10 of the construct to create pJJ429 (Fig. 1 A). Transgenic animals carrying pJJ429 show strong GFP activity only in body wall muscle cells (Fig. 8 A). No activity was seen in the pharynx, intestine, or gonad. Therefore, rescue of *emb-9* (*g23cg45*) mutant animals is possible by expression of functional *emb-9* solely in body wall muscle cells.

Embryos homozygous for *emb-9*(*g23cg45*) do not stain with anti-EMB-9 antisera and show only intracellular accumulation, no basement membrane stain, with anti-LET-2 antisera (20a). We stained pJJ427-rescued *emb-9* (*g23cg45*) embryos with anti-EMB-9 antisera and found that EMB-9 is present in pharyngeal, intestinal, and gonadal basement membranes and under body wall muscles (Fig. 8, B–D). This result further demonstrates that type IV collagen produced in body wall muscle cells can assemble into basement membranes covering other tissues, and

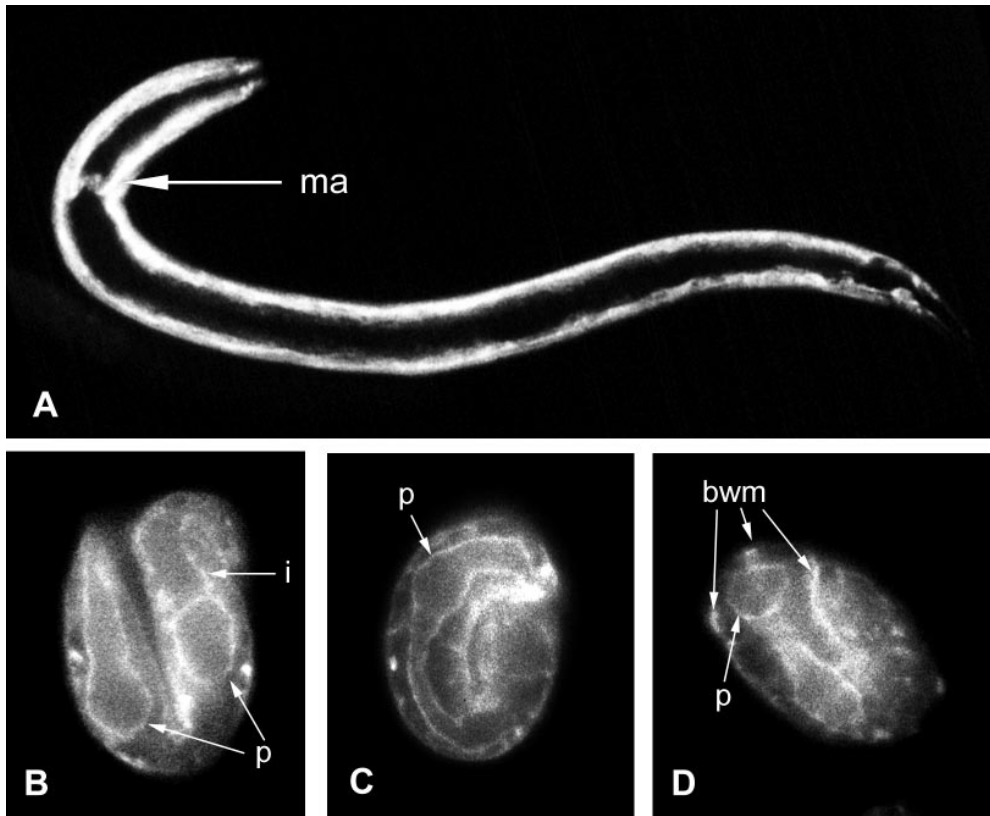


Figure 8. Transgenic rescue of *emb-9(g23cg45)* embryos with an *unc-54* promoter:*emb-9* construct. (A) An L2 larva carrying the *unc-54:emb-9::GFP* reporter pJJ429. The GFP activity is seen only in body wall muscle cells. Muscle arm projections to the nerve ring are indicated (*ma*). (B–D) Homozygous *emb-9(g23cg45)* embryos carrying the *unc-54 promoter:emb-9* construct pJJ427 show anti-EMB-9 staining of basement membranes covering the pharynx (*p*), intestine (*i*), and gonad and under body wall muscle quadrants (*bwm*). In the absence of the rescuing construct, embryos that are homozygous for the putative null mutation *emb-9(g23cg45)* show no staining with anti-EMB-9 antisera.

that it can do so in the absence of any other source of type IV. It also shows that type IV collagen derived from body wall muscles is sufficient for development of *C. elegans* to the adult stage and for at least partial fertility.

Discussion

We have used specific antisera to localize the $\alpha 1$ and $\alpha 2(IV)$ collagen chains of *C. elegans*, EMB-9 and LET-2. Type IV collagen was first detectable within body wall muscle cells of *C. elegans* embryos just beginning morphogenesis. Consistent with this result, the temperature-sensitive periods for both type IV collagen genes, *emb-9* and *let-2*, also begin at this time (23,40; Kramer, J., unpublished results). Soon after, at the comma stage, secreted type IV collagen begins to form stripes under the body wall muscle quadrants. At approximately the same time, type IV collagen first appears around the primordial pharynx and intestine. By the end of embryogenesis, type IV collagen underlies the body wall muscle cells and surrounds the pharynx, intestine, and gonad primordium. The same general pattern of type IV collagen localization continues in larval and adult animals.

Antisera against EMB-9 and LET-2 give the same spatial and temporal staining patterns in wild-type animals, indicating that these chains are colocalized. Additionally, in animals with missense mutations of either chain, the distributions of the chains are altered but still colocalized, and in animals with nonsense mutations in *emb-9*, the LET-2 chain is retained intracellularly and not secreted (20a). The colocalization of EMB-9 and LET-2 and the inability of LET-2 to be secreted in the absence of EMB-9 indicates

that the two chains do not function independently. Since EMB-9 and LET-2 are $\alpha 1$ and $\alpha 2$ -like type IV collagen chains, respectively (19), they may assemble into a single heterotrimeric collagen molecule as do the vertebrate $\alpha 1$ and $\alpha 2$ chains. These results are consistent with the notion that EMB-9 and LET-2 function in a single heterotrimeric type IV collagen molecule.

Basement membranes in two regions of *C. elegans* did not stain with anti-type IV collagen antisera at any stage, those on the hypodermis between the body wall muscle quadrants and those on the pseudocoelomic face of body wall muscles (Fig. 3). The basement membranes in these regions appear ultrastructurally similar to those that cover the intestine and gonad (66) and do stain for type IV collagen. There are three possible explanations for the failure to detect type IV collagen in these regions of basement membrane.

First, it is possible that EMB-9 and LET-2 are present but their epitopes are masked in these regions. Acid-urea extraction, which can uncover masked epitopes in vertebrate basement membranes (69), of whole animals or frozen sections of *C. elegans* had no effect on the type IV collagen staining pattern. All four type IV antisera fail to stain these regions in animals that are fixed with paraformaldehyde or methanol/acetone and in frozen sections. These results make the possibility of epitope masking unlikely and support the idea that EMB-9 and LET-2 are absent from these regions.

Second, it is possible that there is type IV collagen present that is produced by an unidentified gene(s). Southern blot analyses indicated that *emb-9* and *let-2* are likely to be the only type IV collagen gene in *C. elegans* (19). At

this time, with approximately one-half of the *C. elegans* genome sequence complete (61), no other type IV collagen genes have been identified. Multiple cDNA clones derived from *emb-9* and *let-2* have been identified by *C. elegans* EST database projects (62; Kohara, Y., personal communication), but no other type IV collagen transcripts have been seen. Although it is not yet possible to exclude the existence of another type IV collagen gene(s) in *C. elegans*, the probability is small.

Finally, it is possible that there is no type IV collagen in these regions of basement membrane. Although basement membranes can vary in the collagen isoforms they contain (37, 48), they all appear to contain some type IV collagen. There is evidence that during angiogenesis, newly forming basement membranes are laminin rich and lack type IV collagen (15), and there are cell lines that secrete matrix lacking type IV collagen (5, 16). However, there are no reports of fully formed in vivo basement membranes that do not contain type IV collagen. The regions in *C. elegans* on the surface of the hypodermis between body wall muscle quadrants and on the pseudocoelomic face of body wall muscle cells may be basement membrane-like structures that lack type IV collagen.

Whichever of the above possibilities is true, it is clear that the composition and/or modifications of the basement membranes between body wall muscle quadrants and on the pseudocoelomic face of muscles must be different from the other basement membranes in *C. elegans*. Another basement membrane component, the UNC-52 perlecan homologue of *C. elegans*, is also not detected in these same regions of basement membrane (42).

We have identified the cells that express the *emb-9* and *let-2* type IV collagen genes using reporter constructs in

transgenic animals. With minor exception, the type IV collagen-expressing cells in *C. elegans* are mesodermal. The mesoderm of *C. elegans* is derived from four founder cells, MS, D, Cap, and Cpp (54, 55). The D, Cap, and Cpp founders generate only body wall muscle cells, all of which express type IV collagen. The MS cell generates body wall muscles and several other type IV collagen-expressing cell types (Fig. 9). Notably, all of the embryonic MS-derived cells express type IV collagen, except the pharyngeal cells. Type IV collagen expression is also confined to mesodermal cells in *Drosophila* (25, 32, 39) and sea urchin (9, 63). In vertebrates, type IV collagen expression has been reported in both mesenchymal and epithelial cells of several tissues (34, 49, 57, 58), although it is restricted to the mesenchyme in mouse intestine (52).

The combination of antibody localization and gene reporter results indicated that the pharynx and intestine do not express the $\alpha 1$ or $\alpha 2(\text{IV})$ genes, *emb-9* or *let-2*, even though these tissues are covered by basement membranes that contain their products. Also, type IV collagen antisera stain the entire surface of the gonadal basement membrane, even though only a few cells located at the distal and proximal ends of the gonad express the type IV collagen genes. We demonstrated that $\alpha 1(\text{IV})$ chains synthesized in body wall muscle can assemble into basement membranes on these other tissues. Additionally, we found that expression of functional type IV collagen only in body wall muscles is sufficient for *C. elegans* to complete development and be at least partially fertile.

These results raise the question of how the sites of type IV collagen assembly into basement membranes are determined. Presumably, body wall muscle cells secrete type IV collagen from both their hypodermal and pseudocoelomic

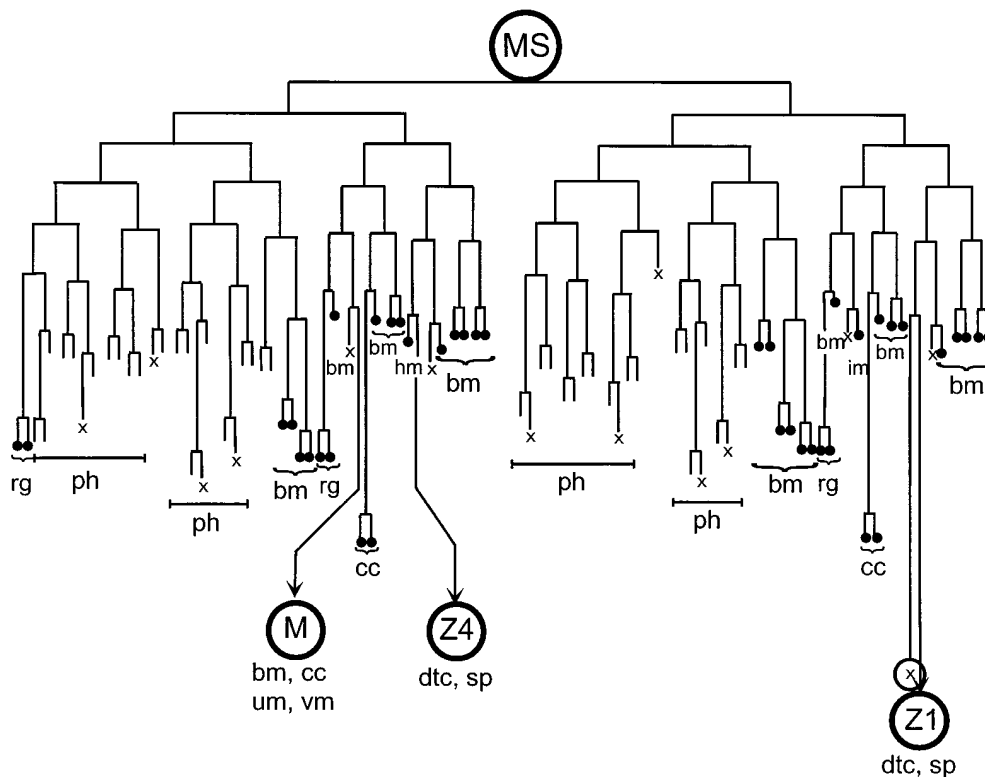


Figure 9. Diagram of the embryonic MS blastomere lineage showing type IV collagen-expressing cells. Cells that express type IV collagen are shown as filled circles. Cells that divide during larval development (*M*, *Z1*, *Z4*) are circled at the bottom of the figure, and their progeny that express type IV collagen are indicated. Cell fates are indicated as: *bm*, body wall muscle; *cc*, coelomocyte; *dtc*, distal tip cell; *hm*, head mesodermal cell; *im*, intestinal muscle; *ph*, pharynx; *rg*, ring ganglion; *sp*, spermatheca; *um*, uterine muscle; *vm*, vulval muscle. Several body wall muscle cells derived from the C and D blastomeres and one body wall, one intestinal, and two anal muscles derived from the AB blastomere also express type IV collagen. The lineage was modified from Sulston et al. (55).

faces. Body wall muscle cells are closely apposed to the hypodermis, and collagen secreted into the small intervening space could assemble into the stripes seen beneath the body wall muscle quadrants. The type IV collagen that assembles into the pharyngeal, intestinal, and gonadal basement membranes is likely to be secreted from the pseudocoelomic face of the muscle cells. Notably, the basement membranes that cover the pseudocoelomic face of body wall muscle cells do not contain detectable type IV collagen. Since the body wall muscle quadrants extend from the head to the tail of the animal, secretion into and diffusion within the pseudocoelomic cavity could provide the necessary type IV collagen for all other tissues.

The remaining question is why all basement membranes that are exposed to the pseudocoelomic cavity do not contain type IV collagen? As noted above, type IV collagen is not detectable in the basement membranes on the pseudocoelomic face of muscle cells or on the hypodermis between body and wall muscle quadrants. Either something is present in these regions that blocks type IV collagen assembly, or something that is required to promote assembly is absent from these regions. We favor the latter possibility, since assembly could be promoted by simply increasing the local concentration of type IV collagen, while no inhibitors of assembly are known.

Type IV collagen can assemble into a polygonal lattice in vitro in a concentration-dependent manner (70). However, the in vivo concentration of type IV collagen may be too low to allow such unassisted assembly. Higher local concentrations of type IV collagen could be achieved if it bound to cell surface receptors or other cell-associated basement membrane components. Likely candidates for this function are integrins and/or nidogen, both of which have been identified in *C. elegans* (18; Kramer, J., unpublished results). Binding to these molecules could produce local concentrations of type IV collagen high enough for assembly to occur. Thus, the localization of type IV collagen may be determined by the localization of cell-associated molecules to which it binds.

The sources and final locations of type IV collagen have been determined in some vertebrate systems. In cocultures of mouse epithelium with chick endothelium, the developing kidney glomerular basement membrane was shown to have type IV collagen contributed by both species (49). In cocultures of fetal intestinal chick mesenchyme with rat endoderm, the resulting subepithelial basement membrane was found to have type IV collagen derived only from the mesenchyme (52, 53). In cocultures of bovine keratinocytes and human fibroblasts, both cell types contributed type IV collagen to the dermal-epidermal basement membrane (34), while nidogen was derived only from fibroblasts (14). So, the type IV collagen in a basement membrane can be derived from cells on either or both sides of it. In these cases, the two groups of cells contributing to basement membrane formation are generally present along a continuous interface. In *C. elegans*, the situation is different because the body wall muscle cells that produce type IV collagen are not immediately adjacent to all sites at which it assembles (Fig. 3). However, the localized assembly of type IV collagen in both the vertebrate and *C. elegans* studies suggest that it must interact with other molecules to direct its assembly.

The assembly properties of the perlecan homologue of *C. elegans*, UNC-52, are different from those of type IV collagen. UNC-52 is deposited in the basement membrane close to its sites of synthesis (42). The tissues that have UNC-52 in their basement membranes all express the *unc-52* gene. Laser ablation of body wall muscle cells results in a hole in the UNC-52 antibody staining pattern where the cells are missing, indicating that UNC-52 does not move far from its site of synthesis. In *C. elegans*, two basement membrane components assemble in different ways; perlecan is deposited locally, while type IV collagen can assemble at sites distant from its site of synthesis.

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