# Characterization of α1(IV) Collagen Mutations in *Caenorhabditis elegans* and the Effects of α1 and α2(IV) Mutations on Type IV Collagen Distribution

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***Abstract.*** Type IV collagen is a major component of basement membranes. We have characterized 11 mutations in *emb-9*, the α1(IV) collagen gene of *Caenorhabditis elegans*, that result in a spectrum of phenotypes. Five are substitutions of glycines in the Gly-X-Y domain and cause semidominant, temperature-sensitive lethality at the twofold stage of embryogenesis. One is a glycine substitution that causes recessive, non–temperature-sensitive larval lethality. Three putative null alleles, two nonsense mutations and a deletion, all cause recessive, non–temperature-sensitive lethality at the threefold stage of embryogenesis. The less severe null phenotype indicates that glycine substitution containing mutant chains dominantly interfere with the function of other molecules. The *emb-9* null mutants do not stain with anti–EMB-9 antisera and show intracellular accumulation of the α2(IV) chain, LET-2, indicating that LET-2 assembly and/or secretion requires EMB-9. Glycine substitutions in either EMB-9 or LET-2 cause intracellular accumulation of both chains. The degree of intracellular accumulation differs depending on the allele and temperature and correlates with the severity of the phenotype. Temperature sensitivity appears to result from reduced assembly/secretion of type IV collagen, not defective function in the basement membrane. Because the dominant interference of glycine substitution mutations is maximal when type IV collagen secretion is totally blocked, this interference appears to occur intracellularly, rather than in the basement membrane. We suggest that the nature of dominant interference caused by mutations in type IV collagen is different than that caused by mutations in fibrillar collagens.

Basement membranes are specialized sheets of extracellular matrix that separate groups of cells from each other and/or from underlying interstitial matrix. Type IV collagen forms a complex branched network that is a major component of basement membranes (Yurchenco and Schittny, 1990; Kuhn, 1994). We are using the nematode *Caenorhabditis elegans* as a model system to study the roles of type IV collagen in basement membrane function, as well as to examine the assembly of basement membrane molecules in vivo. Homologues of several basement membrane proteins, including type IV collagen, perlecan, nidogen/entactin, laminin, and SPARC, have been identified in *Caenorhabditis elegans* (Kramer, 1994; Kramer, J., unpublished results). The strong evolutionary conservation of these molecules indicates that many aspects of basement membrane structure and function have been conserved between nematodes and mammals.

The predominant form of type IV collagen is a heterotrimer containing two α1(IV) chains and one α2(IV) chain (Yurchenco and Schittny, 1990; Kuhn, 1994). The largest portion of the type IV collagen molecule is the central Gly-X-Y repeat domain, which folds into a triple-helical structure. The Gly-X-Y domain contains ~20 interruptions of the Gly-X-Y repeats, which provide flexibility to the molecule. The amino-terminal 7S domain and the globular carboxyl-terminal NC1 domain contain several conserved cysteine residues that participate in intra- and intermolecular disulfide bonding. Formation of the type IV collagen network in basement membranes involves dimerization of NC1 domains and tetramerization of 7S domains, as well as lateral interactions along the triple-helical domain.

In mammals, six type IV collagen genes, encoding α1–α6(IV) chains, have been identified (Hudson, et al., 1993; Kuhn, 1994). These chains are known to assemble into $(\alpha1)_2\alpha2$ and $(\alpha3)_2\alpha4$ heterotrimers, but it is not clear how α5 and α6 chains assemble. While the α1 and α2 chains are present in all basement membranes, the other chains have restricted tissue distributions, being most abundant in the kidney (Langeveld et al., 1988; Sanes et al., 1990; Kashtan and Kim, 1992; Ninomiya et al., 1995).

Two type IV collagen genes have been characterized in

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*C. elegans* (Guo and Kramer, 1989; Sibley et al., 1993, 1994) and sea urchin (Exposito et al., 1993, 1994), and a single gene in *Drosophila* (Blumberg et al., 1987), *Ascaris* (Pettitt and Kingston, 1991), and *Brugia* (Caulagi and Rajan, 1995). In both *C. elegans* and sea urchin, one type IV collagen gene is α1-like and the other is α2-like, suggesting that their products may form $(\alpha1)_2\alpha2$ heterotrimers. No more than two type IV collagen genes have been identified in any invertebrate, and there is evidence that only two type IV collagen genes exist in *C. elegans* (Guo and Kramer, 1989).

The genetic designations of the α1(IV)-like and α2(IV)-like genes of *C. elegans* are *emb-9* and *let-2*, respectively. Ultrastructural analyses have identified basement membranes covering the pseudocoelomic face of the hypodermis and surrounding the pharynx, intestine, gonad, and body wall muscles of *C. elegans* (Albertson and Thomson, 1976; White et al., 1976; White, 1988). Using chain-specific antisera, the EMB-9 and LET-2 chains of *C. elegans* were found to colocalize and be present in all of these basement membranes, except those on the pseudocoelomic face of body wall muscles and the region of the hypodermis between body wall muscle quadrants (Graham et al., 1997). Thus, the EMB-9 and LET-2 chains are most similar to the mammalian α1 and α2(IV) chains both structurally and in having a wide tissue distribution.

Mutations have been identified in the α3, α4, and α5(IV) genes of human (Lemmink et al., 1994; Mochizuki et al., 1994; Antignac 1995; Tryggvason, 1995), the α5(IV) gene of dog (Zheng et al., 1994), the α3(IV) gene of mouse (Cosgrove et al., 1996; Miner and Sanes, 1996), and the α1 and α2(IV) genes of *C. elegans* (Guo and Kramer, 1989; Sibley et al., 1993, 1994). The mutations in the mammalian α3–α5(IV) genes can cause Alport syndrome, a progressive glomerulonephritis with variably associated eye and ear defects. The primarily renal focus of Alport syndrome is consistent with the fact that the α3–α5 chains are most abundant in the kidney glomerulus. Antibody staining of kidney samples from patients with mutations in the α3, α4, or α5(IV) gene shows that all three chains are absent, suggesting that they are interdependent for normal assembly and/or deposition into the basement membrane (Peissel et al., 1995). Mutations have not been identified in the mammalian α1 or α2(IV) genes.

Mutations have been identified in the *C. elegans emb-9* and *let-2* genes, homologues of the mammalian α1 and α2(IV) genes (Guo et al., 1991; Sibley et al., 1994). Mutations in both genes can cause arrest during embryonic development, indicating that there is a requirement for type IV collagen function in *C. elegans* embryogenesis. The molecular and phenotypic characteristics of 17 mutations of *let-2* have been analyzed (Sibley et al., 1994). 15 of the mutations were found to be substitutions for glycine residues in the Gly-X-Y domain and to cause a wide range of phenotypes. The mildest of these were nearly wild-type at 15 and 20°C but embryonic lethal at 25°C. The most severe allele was embryonic lethal at all temperatures. The most severe phenotype was arrest at the twofold stage of embryonic development, but larval arrest and adult sterility were also seen, particularly at intermediate temperatures. Three mutations in the *emb-9* gene were identified as glycine to glutamate substitutions (Guo et al., 1991). These three mutations result in very similar temperature-sensitive phenotypes, with twofold arrest as their most severe effect.

In this study, we have molecularly and genetically characterized 11 *emb-9* mutations that produce a range of phenotypes. We show that null mutations of *emb-9* cause a less severe phenotype than most missense mutations. Using chain-specific antisera, we show that missense mutations in either chain cause intracellular accumulation of both chains and that the degree of intracellular accumulation is correlated with the severity of the phenotype. In *emb-9* null mutants, the LET-2 chain is shown to accumulate intracellularly and not be secreted.

## Materials and Methods

### Nematode Strains

*C. elegans* strains were maintained and handled as described (Brenner, 1974). The *C. elegans* Bristol strain N2 has been designated as wild type. Animals homozygous for the *emb-9* alleles *g23*, *g34*, *b117*, *b189*, and *hc70* (Miwa et al., 1980; Wood et al., 1980; Cassada et al., 1981) were maintained at 15°C. The *emb*-9 alleles *st540*, *st545* (Williams and Waterston, 1994), *cg45*, *cg46*, *cg56*, and *cg57* (see below) were maintained as heterozygotes with *qC1*, a balancer chromosome that prevents recombination in the region of *emb-9* (Edgley et al., 1995). The following *let-2* alleles were used: *g25, g30* (Miwa et al., 1980; Cassada et al., 1981), *b246* (Wood et al., 1980), *mn109*, *mn129*, and *mn139* (Meneely and Herman, 1979).

### Generation of emb-9 Alleles

The *emb-9(cg56)* allele was generated using the strain *unc-32(e189)/qC1; cgEx1[emb-9(+)rol-6(su1006)]*. The *unc-32* marker is tightly linked to *emb-9* and used to identify chromosome III. *cgEx1* is an extrachromosomal array carrying the wild-type *emb-9* gene and *rol-6(su1006)*, which confers a right roller phenotype (Mello et al., 1991; Kramer and Johnson, 1993). The strain was mutagenized with ethyl methanesulfonate (EMS) and grown at 15°C. F1 Rol non-Unc animals were isolated onto separate plates and shifted to 25°C. Plates were screened for the presence of Rol Unc and the absence of non-Rol Unc F2 progeny. One allele, *cg56*, was isolated and backcrossed twice using the following scheme: The *unc-32 emb-9(cg56)/qC1; cgEx1* animals were crossed with N2 males, and cross progeny males were individually crossed into *unc-32/qC1* hermaphrodites. Offspring from this cross that produced approximately one-quarter lethal and one-quarter *qC1* progeny, indicating they were *unc-32 emb-9(cg56)/qC1*, were selected and maintained.

Putative null mutations were generated using the strain *unc-36(e251) emb-9(g23); qDp3. qDp3* is a free duplication that carries *unc-36(+)* and *emb-9(+)*. The strain does not survive at 25°C because of the semidominance of *emb-9(g23)*. The strain was mutagenized with EMS and grown at 15°C for two generations. The F2s were shifted to 25°C and animals that survived were isolated onto separate plates. Three alleles, *cg45*, *cg46*, and *cg57*, were isolated and backcrossed four times as follows: *unc-36 emb-9(cg45, cg46, or cg57); qDp3* animals were mated with N2 males, and the resulting males were crossed individually into *unc36/qC1* hermaphrodites. F1 cross progeny that produced approximately one-quarter lethal and one-quarter *qC1* progeny, indicating that they were *unc-36 emb-9/qC1*, were selected.

### Phenotypic Characterization of emb-9 Alleles

*emb-9* mutants were scored for viability at 15, 20, and 25°C. Animals heterozygous for *emb-9* were generated by crossing the mutant strains with wild-type males. The heterozygotes were placed individually onto separate temperature-equilibrated plates. Eggs laid within the next 6–7 h (25°C), 8–10 h (20°C), or 14–16 h (15°C) were counted, and the parent was removed. 1 d later, plates were examined to determine the numbers of eggs that had hatched, or that failed to hatch (Emb). Plates were scored again after 3–4 d (25°C), 5–6 d (20°C), or 7–8 d (15°C) to determine the number of animals that had developed to adults (Ad). Animals that were severely abnormal and sterile as adults were noted (Ste). Larvae not accounted for as adults were considered to have arrested during larval de-

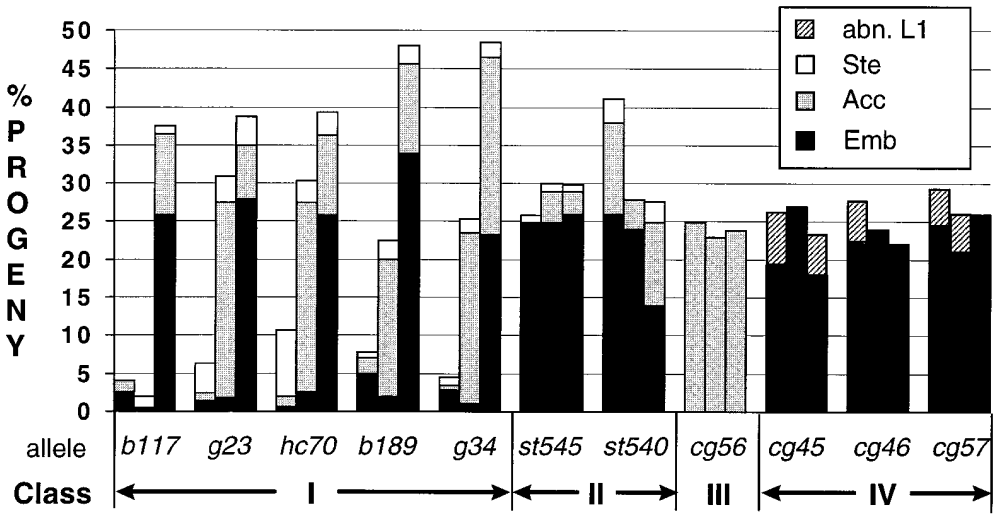


*Figure 1.* Phenotypes of offspring from *emb-9/+* mothers. The percentage of progeny derived from *emb-9/+* heterozygote mothers that arrested during embryogenesis (*Emb*), arrested as larvae (*Acc*), became sterile adults (*Ste*), or hatched as severely deformed L1s (*abn. L1*) are indicated. Each allele was tested at 15°C (*left bars*), 20°C (*middle bars*), and 25°C (*right bars*). The 11 *emb-9* alleles have been divided into four phenotypic classes (I–IV) based on their temperature sensitivity, dominance, and predominant stage of arrest. Completely recessive *emb-9* alleles would be expected to produce 25% or less abnormal progeny, while semidominant alleles could result in up to 75% abnormal progeny. At least 200 progeny were examined for each allele at each temperature.

velopment (Acc). The number of Acc animals could be reliably inferred since the N2, *unc-32*, and *unc-36* controls gave very low percentages of Acc larvae at all temperatures. The total Emb and Acc for N2, *unc-32*, and *unc-36* were from 0–3%. These background values have been subtracted from the results obtained from the relevant *emb-9* alleles linked to *unc-32* or *unc-36.* At least 200 embryos were scored for each strain at each temperature.

## Complementation Tests

*st545* and *st540* were previously identified as alleles of *emb-9* by testing for complementation with the *emb-9(hc70)* allele (Williams and Waterston, 1994). We retested for complementation using the putative null allele *emb-9(g23cg45). unc-36 emb-9(g23cg45)/qC1* hermaphrodites were crossed with N2 males, and single cross progeny males were mated with *st545/qC1* and *st540/qC1.* The production of one-quarter embryonic lethal progeny from these crosses indicated failure of *st545* or *st540* to complement *emb-9(g23cg45).* The presence of viable cross progeny males indicated that the mating was successful. The absence of *qC1* homozygous cross progeny indicated that the male was of the genotype *unc-36 emb-9(g23cg45)/+ +*.

## Identification of Nucleotide Alterations in emb-9 Alleles

Genomic DNAs were prepared from *emb-9* mutant strains and the wild-type N2 strain as previously described (Kramer et al., 1988). *g23*, *g34*, *hc70*, *b117*, and *b189* carrying strains were grown as homozygotes at 15°C, and *st540*, *st545*, and *cg56* were maintained as heterozygotes balanced across from *qC1*. The *cg45*, *cg46*, and *cg57* alleles were maintained as *unc-36 emb-9*; *qDp3* strains.

Nucleotide alterations were identified using either the chemical mismatch method or by directly sequencing the entire *emb-9* gene. PCR fragments (1–2 kb) of wild-type and *emb-9* mutant DNAs were prepared by incubating genomic DNA (500–700 ng) with 1× PCR buffer (GIBCO BRL, Gaithersburg, MD), 2 mM $MgCl_2$, 2.5 ng/ml BSA, 200 μM dNTPs, and 2.5 U Taq polymerase (GIBCO BRL) in a total volume of 100 μl. PCR samples were incubated at 94°C for 1 min, 47–63°C (depending on $T_m$ of primers) for 1 min, and 72°C for 1 min. This temperature cycle was repeated 30 times in an automated temperature cycler. PCR fragments were generated so as to overlap one another by 200–300 bp. Six 1–2-kb fragments were used to span the *emb-9* gene, including 484 bp 5′ of the start codon and 111 bp 3′ of the stop codon. PCR fragments were purified using Geneclean (Bio 101 Inc., La Jolla, CA). Nucleotide alterations in these fragments were identified using the chemical mismatch detection procedure (Montandon et al., 1989). Point mutations were identified by direct sequencing of PCR fragments in the areas of mismatch, using Sequenase (United States Biochemical Corp., Cleveland, OH) and a modified annealing procedure (Kusukawa et al., 1990).

Several mutations were identified by directly sequencing overlapping PCR fragments that span the entire *emb-9* gene. PCR fragments were cleaned using Centricon 100 columns (Amicon, Beverly, MA). 200–300 ng of template DNA was used for Taq DyeDeoxy terminator thermal cycle sequencing (Appiled Biosystems, Inc., Foster City, CA). Samples were spun through spin columns (model Centri-Sep; Princeton Separations Inc., Adelphia, NJ) and dried in a vacuum centrifuge. Each sample was resuspended in 4 μl of formamide-EDTA solution (83% deionized formamide, 8 mM EDTA, pH 8.0). Samples were denatured at 90°C for 2 min, transferred immediately onto ice, and loaded onto a 6% polyacrylamide gel on an automated sequencer (model 373A; Applied Biosystems, Inc.).

## Immunofluorescent Staining of C. elegans Embryos

Animals carrying temperature-sensitive alleles, *emb-9(g23*, *g34*, *hc70*, *b117*, *b189)* and *let-2(b246*, *g25*, *g30*, *mn109*, *mn129*, *mn139)*, were maintained as homozygotes at 15°C. To collect embryos raised at higher temperatures, plates containing a mixed population were shifted from 15 to 20 or 25°C and allowed to lay eggs overnight.

Embryos were isolated from animals by alkaline hypochlorite (5:3:1 water/4M NaOH/bleach) treatment for 7–10 min. The eggs were washed several times with PBS, fixed with 3% paraformaldehyde in PBS for 15 min at room temperature, rinsed with distilled water ($dH_2O$) and then stored at −80°C in 100% methanol for 30 min to 7 d.

Fixed embryos were rehydrated, treated with 6 M urea, 0.1 M glycine, pH 3.5, for 15 min at room temperature, rinsed with $dH_2O$, and blocked for 1 h in PBS containing 0.5% Tween-20 (PBS-T) and 10% normal donkey serum (NDS)[1]. Primary antibodies against *C. elegans* type IV collagen and myosin heavy chain B were added in PBS-NDS for 1 h at 37°C. The embryos were then washed three times for 15 min each with PBS-T and incubated for 1 h at 37°C with secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and 0.1 mg/ml diamidinophenolindole in PBS-T–NDS, washed 3× for 15–30 min with PBS-T, and then mounted in DABCO medium (20% glycerol, 10% polyvinyl alcohol, 2.5% DABCO in 40 mM Tris-HCL, pH 8.3) with phenylenediamine freshly added to 1 mg/ml.

The chain-specific anti–type IV collagen antisera used in this study have been described (Graham et al., 1997). Affinity-purified antiserum NW1910 was used to identify the α1(IV) chain EMB-9, and affinity-purified NW68 antiserum was used to identify the α2(IV) chain LET-2. The 5-8 mouse monoclonal antibody against *C. elegans* myosin heavy chain UNC-54 (Miller et al., 1983) was used to stain body wall muscle cells. The primary antibodies were detected using fluorescein-conjugated donkey anti–rabbit and/or rhodamine-conjugated donkey anti–mouse IgG secondary antibodies.

1. *Abbreviation used in this paper*: NDS, normal donkey serum.

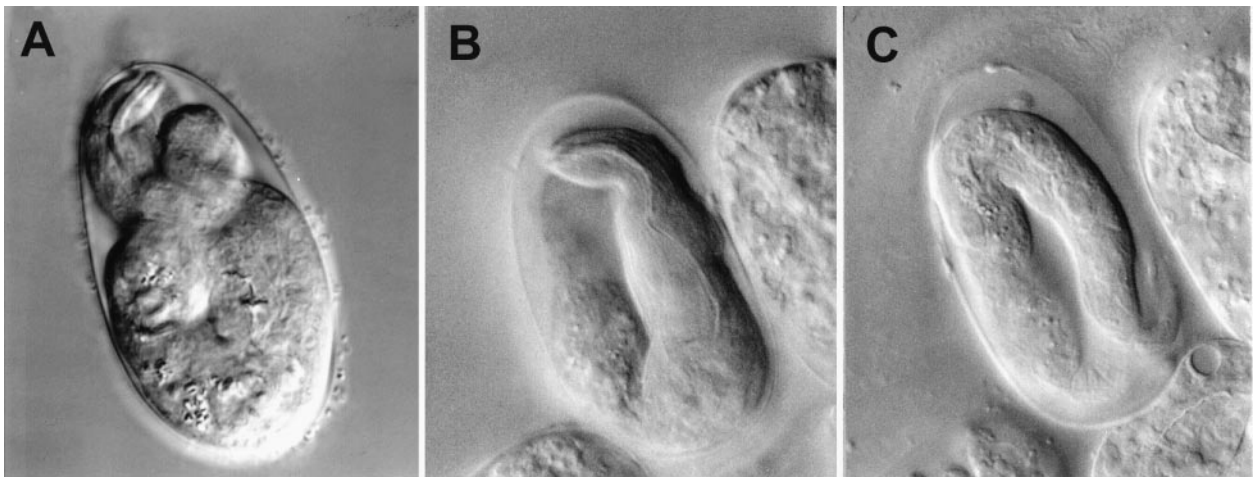


*Figure 2.* Differential interference contrast micrographs of *emb-9* mutant embryos grown at 25°C. (*A*) *emb-9(g23)* mutant embryo arrested at the twofold stage of embryogenesis. (*B* and *C*) Two focal planes of an *emb-9(g23cg46)* mutant embryo arrested at the threefold stage of embryogenesis.

## Results

### Phenotypic Characterization of emb-9 Mutants

Eight alleles of *emb-9* were characterized for their effects at 15, 20, and 25°C. Progeny from heterozygous, *emb-9/+*, mothers were scored for those that arrested during embryogenesis (Emb) or larval development (Acc), or became sick, sterile adults (Ste) (Fig. 1). For recessive alleles, 25% or fewer animals should show abnormal phenotypes, while semidominant alleles could result in up to 75% abnormal animals. These *emb-9* alleles were grouped into three classes based on their general phenotypic characteristics.

The class I alleles, *b117*, *g23*, *hc70*, *b189*, and *g34*, are all strongly temperature sensitive. At 15°C, there is a very low level of lethality or sterility. At 20°C, there is a large increase in larval lethality in all except the *b117* allele. The vast majority of these larvae arrest at the L1 or L2 stages. At 25°C, there is approximately 25% embryonic lethality for each of the alleles, indicating that essentially all homozygous mutant animals arrest as embryos. This result is consistent with the 100% embryonic lethality seen when homozygous embryos are shifted to 25°C (Cassada et al., 1981; Kramer, J., unpublished results). Larval lethality and sick, sterile adults increase the total percentages of abnormal animals to 37 to 48%, indicating that each of these alleles is semidominant.

The class II alleles, *st545* and *st540*, show high levels of embryonic lethality and mild temperature sensitivity. *st545* is essentially a recessive, embryonic lethal. It shows ~25% embryonic arrest at all temperatures and a very low level of larval arrest at 20 and 25°C. *st540* appears to be mildly cold sensitive, displaying decreased embryonic lethality at 25 versus 15°C and some semidominant larval arrest at the lower temperature.

The class III allele, *cg56*, is a recessive, non–temperature-sensitive larval lethal. All *cg56* homozygotes arrest during the L1–L2 larval stages at all temperatures. There is no apparent semidominance of this allele.

### Generation and Characterization of Putative Null Alleles of emb-9

Most *emb-9* alleles show semidominant effects, which are likely to result because mutant protein interferes with the functions of other molecules. Complete absence of EMB-9 would eliminate any dominant interfering effects and allow determination of the phenotype that results when the α1(IV) collagen chain is missing. For this purpose, a genetic screen was designed to isolate putative null mutations of the *emb-9* gene.

Three putative *emb-9* null alleles were generated by screening for mutations that eliminate the temperature-sensitive dominant lethality of *emb-9(g23).* A strain carrying two copies of *g23* and a wild-type copy of *emb-9* on a duplication, *unc-36 emb-9(g23); qDp3[emb-9(+)]*, is viable and fertile at 15°C but dies at 25°C. This strain was mutagenized and screened for F2 animals that could survive and reproduce at 25°C, and three putative null alleles, *cg45*, *cg46*, and *cg57*, were identified. All three of these alleles cause less severe phenotypes than *g23. g23* homozygotes grown at 25°C arrest at approximately the twofold length of embryogenesis, when the head and tail of the elongating embryo have just reached each other (Fig. 2 *A*). Arrested embryos show extensive herniations of the body wall. In contrast, *cg45*, *cg46*, and *cg57* embryos elongate further, to at least the threefold stage of embryogenesis (Fig. 2, *B* and *C*). The putative null mutant animals also display less severe herniations of the body wall.

Phenotypic characterization of the putative null mutations indicates that they are recessive, embryonic lethals and belong to a new phenotypic class, class IV (Fig. 1). Approximately 25% of the progeny from *cg45*, *cg46*, or *cg57* heterozygous mothers arrest as embryos, at the three- to fourfold stage. A small number of embryos hatch, but they are small, herniated, and paralyzed (Fig. 1, *abn. L1*). These larvae do not develop further and appear to be homozygous mutant embryos whose egg shells have ruptured. There is no significant effect of temperature on the phenotype of these mutants, as would be expected for null mutants.

*Table I. Molecular Identification of emb-9 Mutations*

| Allele | Site of mutation* | Amino acid substitution |
|---|---|---|
| *b189* | G 1727 A | Gly 213 Glu |
| *cg56* | G 2266 A | Gly 359 Arg |
| *g34* | G 2396 A | Gly 402 Glu |
| *g23* | G 2414 A | Gly 408 Glu |
| *hc70* | G 2414 A | Gly 408 Glu |
| *cg46* | Δ4592–5089 | |
| *cg57* | C 4953 T | Gln 1156 Stop |
| *b117* | G 5002 A | Gly 1175 Asp |
| *cg45* | C 5649 T | Gln 1388 Stop |

*Numbering in nucleotides with start of translation designated as +1. (Guo et al., 1991; These sequence data are available from GenBank/EMBL/DDBJ under accession number X56979.)

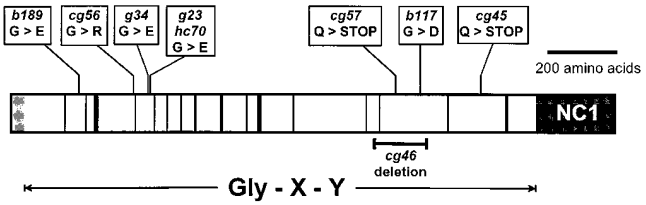


*Figure 3.* Location of the mutations in the *C. elegans* α1(IV) collagen gene *emb-9*. The structure of the α1(IV) collagen chain EMB-9 is shown. The central Gly-X-Y repeat domain is indicated as an open box, with interruptions of the Gly-X-Y repeats shown as vertical bars. The amino- and carboxyl-terminal non–Gly-X-Y domains are shaded. The locations and alterations caused by the mutations are shown above the structure. The extents of the *cg46* deletion mutation are shown by a bar below the structure.

### *Molecular Identification of emb-9 Mutations*

Nine mutations in the *emb-9* gene have been identified, three previously (Guo et al., 1991) and six here, by comparison with the wild-type *emb-9* gene sequence. The precise alterations in the mutants are given in Table I and are shown graphically in Fig. 3. All nine of the mutations are in the triple-helical (Gly-X-Y) repeat domain of the EMB-9 chain. Six of the mutations result in replacement of glycine with aspartate, glutamate, or arginine. Two of the putative null mutations, *cg45* and *cg57*, result in nonsense codons, and one, *cg46*, is a deletion of 497 bp. The *cg46* deletion removes the last 22 nucleotides of intron 9 and 475 nucleotides of exon 10. Failure to splice this intron would result in translation termination after incorporation of three amino acids from intron 9.

Nucleotide alterations were not identified in *st545* or *st540* by either chemical mismatch detection or direct sequencing of mutant genomic DNAs. These mutations were previously assigned to *emb*-9 based on failure to complement the temperature-sensitive allele *emb-9(hc70)* (Williams and Waterston, 1994). We retested complementation of these alleles using the putative null allele *cg45* and found that they do fail to complement (see Materials and Methods), indicating that they are indeed *emb-9* alleles. Since these alleles are maintained as heterozygotes across from the *qC1* balancer chromosome, the wild-type sequence in the background may have interfered with the detection of these mutant alterations.

### *Putative Null Mutations of emb-9 Cause Intracellular Accumulation of LET-2*

We have determined the effects of *emb-9* and *let-2* mutations on type IV collagen distribution by staining mutant embryos with chain-specific antisera. The distributions of EMB-9 and LET-2 in wild-type embryos have been described (Graham et al., 1997) and are outlined here for comparison to the patterns in mutants. Antisera against EMB-9 and LET-2 show identical staining patterns, suggesting that the two chains assemble into a single heterotrimeric collagen molecule. In lima to 1 1/2-fold embryos, strong type IV collagen stain is seen within body wall muscle cells (Fig. 4, *A* and *B*). Beginning in the comma stage, secreted type IV collagen accumulates beneath the body wall muscles and around the forming pharynx and intestine. From the twofold stage on, most type IV collagen stain is extracellular. Both EMB-9 and LET-2 are detected in the region of the hypodermal basement membrane underlying the body wall muscle quadrants and in the basement membranes surrounding the pharynx, intestine, and gonad (Fig. 4, *C–F*). No type IV collagen is detected in the hypodermal basement membrane between the body wall muscle quadrants or on the pseudocoelomic face of the muscles. This pattern is altered by mutations in *emb-9* or *let-2*.

All three putative *emb-9* null mutations have the same effect on type IV collagen localization. Null mutant embryos do not show any detectable staining with EMB-9–specific antisera (Fig. 5, *A* and *B*). They do stain with LET-2–specific antisera (Fig. 5, *C* and *D*); however, all of the stain is intracellular, and no LET-2 is detectable in basement membranes. The intracellular staining pattern is punctate and frequently appears as two strong spots located on opposite sides of each of the body wall muscle cell nuclei. No LET-2 is detectable within hypodermal, intestinal, or pharyngeal cells. This is consistent with previous evidence that type IV collagen gene expression occurs in body wall muscle cells but is undetectable in hypodermal, intestinal, and pharyngeal cells (Graham et al., 1997). The intracellular accumulation of LET-2 in putative *emb-9* null mutants indicates that LET-2, the α2(IV) chain, is not secreted in the absence of full-length EMB-9, the α1(IV) chain.

### *Glycine Substitution Mutations Cause Intracellular Accumulation of Both Type IV Collagen Chains*

Most *emb-9* (6 of 11) and *let-2* (15 of 17) mutations are substitutions for glycine residues in the Gly-X-Y repeat domain of the collagen. We stained the five *emb-9* and twelve *let-2* temperature-sensitive glycine substitution mutants with anti–EMB-9 and anti–LET-2 antisera. In all cases, both antisera gave very similar patterns, indicating that EMB-9 and LET-2 chains are colocalized in these *emb-9* and *let-2* mutants. The primary effect seen was loss of type IV stain from basement membranes and the appearance of strong intracellular stain in body wall muscle cells. The degree of this effect varied depending on the temperature and allele and correlated with the severity of the phenotype. In the most severe allele/temperature combinations,

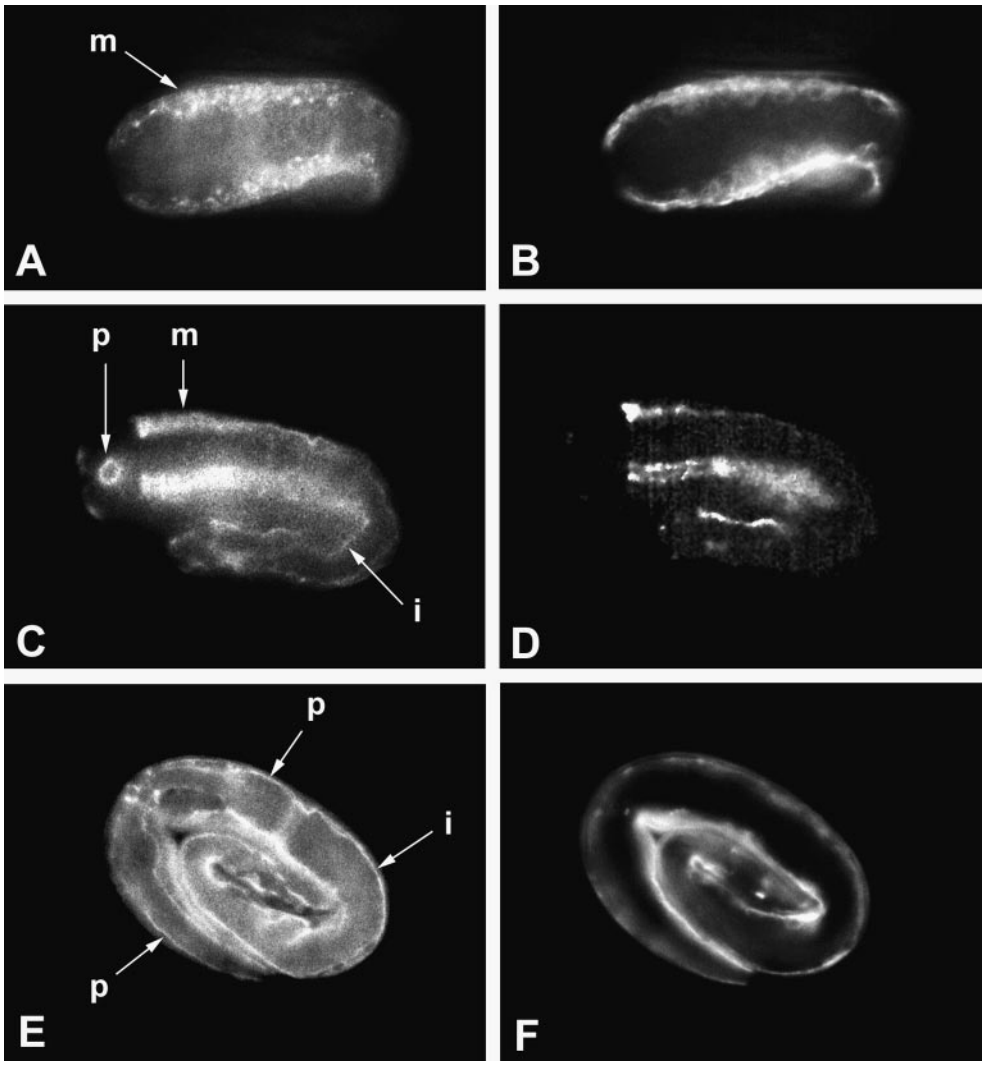


*Figure 4.* Staining of wild-type *C. elegans* embryos with collagen IV antibodies. (*A*, *C*, and *E*) Embryos stained with type IV collagen–specific antisera. (*B*, *D*, and *F*) The same embryos stained with the body wall muscle myosin antibody. (*A*) A 1 1/2-fold embryo stained with the LET-2–specific antiserum. The stain is in body wall muscle cells (*m*) and is primarily intracellular at this stage. (*C*) A threefold stage embryo stained with the EMB-9–specific antiserum. Staining is seen beneath body wall muscles (*m*), surrounding the pharynx (*p*), and on the surface of the intestine (*i*). (*E*) A fourfold stage embryo stained with the LET-2–specific antiserum. The focal plane is near the center of the embryo, so the body wall muscles are not in focus. Staining of the basement membranes around the pharynx (*p*) and intestine (*i*) are indicated.

all type IV collagen was intracellular, while milder combinations showed progressively less intracellular stain and more basement membrane stain.

The *emb-9(g23)* mutant is shown as an example of the pattern seen in the five *emb-9* mutants (Fig. 6). At 25°C, essentially all type IV collagen stain is found as bright spots within body wall muscle cells (Fig. 6, *A* and *B*). There is little or no detectable extracellular staining in basement membranes. At 20°C, strong intracellular stain is still seen, but weak extracellular stain in basement membranes can be seen as well (Fig. 6, *C* and *D*). At 15°C, the intensity of the intracellular stain decreases markedly, and strong extracellular stain of basement membranes is seen (Fig. 6, *E* and *F*). Note that at 15°C, *g23* mutants develop normally, at 20°C most hatch but arrest as early larvae, while at 25°C they arrest at the twofold stage of embryogenesis (Fig. 1).

The *let-2(b246)* mutation causes a milder temperature-sensitive phenotype than *emb-9(g23).* At 15°C, *b246* homozygotes develop normally, at 20°C 78% complete development to adult, while at 25°C they all arrest at the twofold stage of embryogenesis (Sibley et al., 1994). The milder phenotype of *b246* is reflected in the distribution of type IV collagen at different temperatures. At 25°C, type IV collagen accumulates intracellularly, and little or no basement membrane stain is detectable (Fig. 7 *A*). At 20°C, type IV collagen distribution is nearly normal with only a small amount of intracellular accumulation (Fig. 7 *B*). At 15°C, the type IV collagen distribution is wild-type in appearance (Fig. 7 *C*).

Six temperature-sensitive glycine substitution mutants of *let-2* were analyzed by staining embryos raised at 15, 20, and 25°C with LET-2 antiserum. The percentages of embryos that showed strong intracellular stain in body wall muscle cells and that showed strong pharyngeal basement membrane stain were determined (Table II). In general, intracellular body wall muscle stain increased and pharyngeal basement membrane stain decreased with increasing temperature. Thus, as the temperature increases, secretion of mutant type IV collagen is more strongly inhibited. These results indicate that temperature-sensitive glycine substitution mutations in either EMB-9 or LET-2 cause both chains to be retained intracellularly. The degree of intracellular retention and the severity of the phenotype increase with increasing temperature.

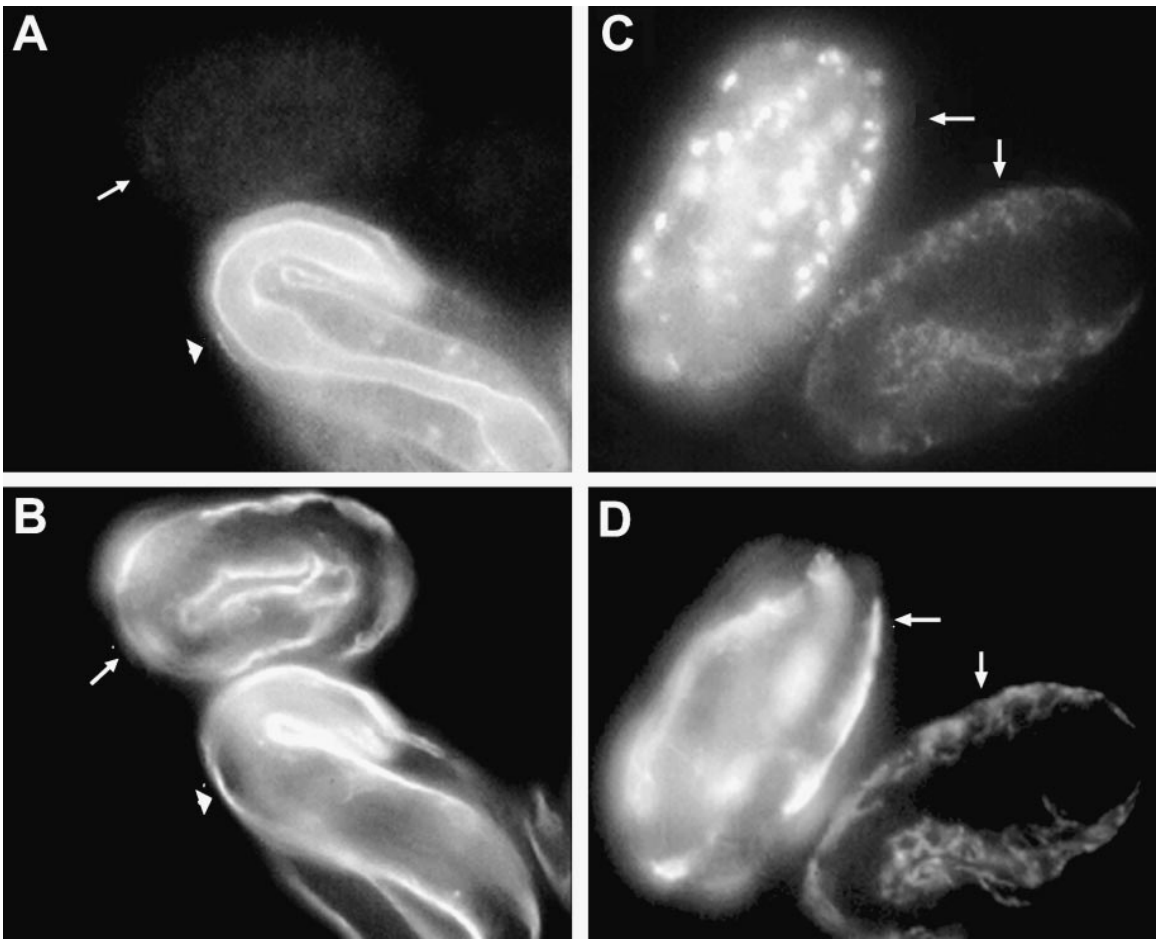


*Figure 5.* Staining of putative *emb-9* null mutants with type IV collagen antibodies. Embryos from *unc-36 emb-9(g23cg45)/qC1* mothers were stained with antisera against EMB-9 (*A*) or LET-2 (*C*), and body wall muscle myosin (*B* and *D*). Homozygous *unc-36 emb-9(g23cg45)* embryos are indicated with arrows, and a heterozygous *unc-36 emb-9(g23cg45)/qC1* embryo is marked with an arrowhead. The heterozygous embryo stains with both the anti–EMB-9 (*A*, *arrowhead*) and antimyosin (*B*) antisera. The homozygous *cg45* embryo fails to stain with the anti–EMB-9 antiserum (*A*, *arrow*), but does stain with the antimyosin antiserum (*B*, *arrow*). Note the disruption of the myosin staining pattern in the homozygous mutant. Two homozygous *cg45* mutant embryos stain with anti–LET-2 antiserum (*C*, *arrows*), but only show intracellular accumulation, no extracellular basement membrane stain. Both of these embryos stain with antimyosin antiserum (*D*), but show a disrupted pattern.

### *A Non–temperature-sensitive Glycine Substitution Mutation*

*emb-9(cg56)* is a glycine substitution mutation that results in a non–temperature-sensitive, recessive larval lethal phenotype (Fig. 1). Staining of this mutant with either EMB-9 or LET-2 antisera shows the same pattern at all temperatures (Fig. 8). Both EMB-9 and LET-2 show strong accumulation in body wall muscle cells, but there is also very weak staining of the basement membranes underlying the body wall muscles. The small amount of extracellular staining in *cg56* mutants is similar to what is seen in *emb-9(g23)* mutants grown at 20°C and is consistent with the fact that *cg56* animals survive to the L1 stage, as do *g23* animals at 20°C. The small amount of type IV collagen present in the basement membranes of *cg56* mutant animals is sufficient to allow completion of embryogenesis.

### *Class II Mutants of emb-9*

In both of the class II mutants, *st545* and *st540*, EMB-9 and LET-2 chains accumulate intracellularly in body wall muscle cells, and there is no detectable extracellular basement membrane stain. However, the pattern of intracellular accumulation in *st545* and *st540* is different from that in the other *emb-9* and *let-2* mutants. The stain in *st545* and *st540* mutants generally appears as a ring surrounding the nucleus (Fig. 9 *A*), while in all other *emb-9* and *let-2* mutants, the intracellular stain generally appears as two spots on opposite sides of the nucleus (Fig. 9 *B* and Figs. 5, 6, and 7). Apparently, the *st545* and *st540* mutations cause accumulation of type IV collagen in a different intracellular compartment within muscle cells than do the other mutations.

### *Absence of Type IV Collagen Causes Body Wall Muscle Disruption*

*emb-9* and *let-2* mutant embryos were costained with an antibody against myosin heavy chain B, which is expressed in body wall muscle cells. The myosin staining served as a

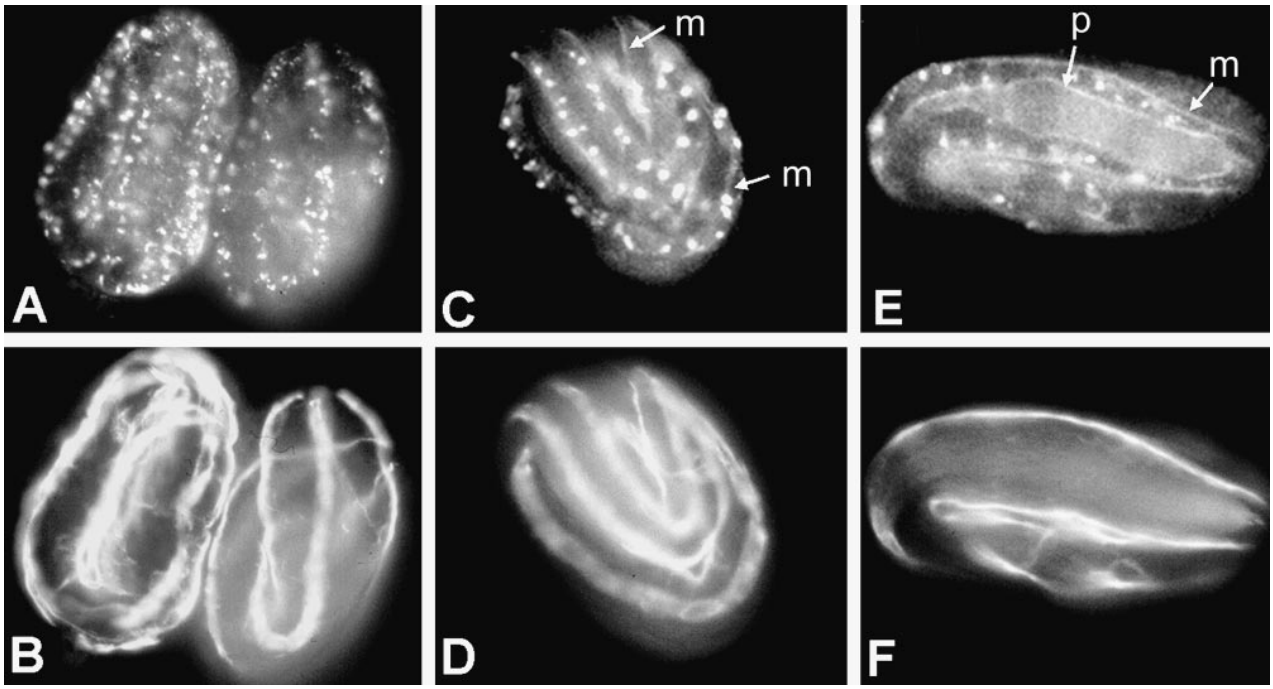


*Figure 6.* Type IV collagen disruption in an *emb-9* temperature-sensitive glycine substitution mutant. Staining of the temperature-sensitive glycine substitution mutant *emb-9(g23).* Embryos grown at 25°C (*A* and *B*), 20°C (*C* and *D*), or 15°C (*E* and *F*) were stained with the anti–LET-2 (*A*, *C*, and *E*) and antimyosin antisera (*B*, *D*, and *F*). Staining with EMB-9 antisera gives a pattern identical to that seen for LET-2. At 25°C (*A* and *B*), type IV collagen appears as intense spots in body wall muscle cells, and there is little or no type IV in the normal extracellular basement membrane locations. At 20°C (*C* and *D*), intracellular accumulation of collagen IV is seen, though often with reduced intensity, and weak staining of the basement membranes separating body wall muscle cells from the hypodermis is detected (*m*). At 15°C (*E* and *F*), intracellular accumulation of type IV collagen is greatly reduced, and strong stain of pharyngeal (*p*), intestinal, and muscle (*m*) basement membranes is seen.

control for permeability of embryos and permitted analysis of the organization of body wall muscles. We had noted previously that the body wall muscle quadrants were disrupted in *emb-9* and *let-2* missense mutants. However, in these mutants it is not clear if disruption is due to the absence of type IV collagen or the dominant interfering effects of abnormal type IV collagen. Analyses of the three putative *emb-9* null alleles indicate that they do cause disruption of the body wall muscle quadrants. In wild-type animals, the four body wall muscle quadrants appear as four stripes extending from head to tail of the animal (Fig. 10 *A*). In temperature-sensitive *emb-9* or *let-2* mutants at

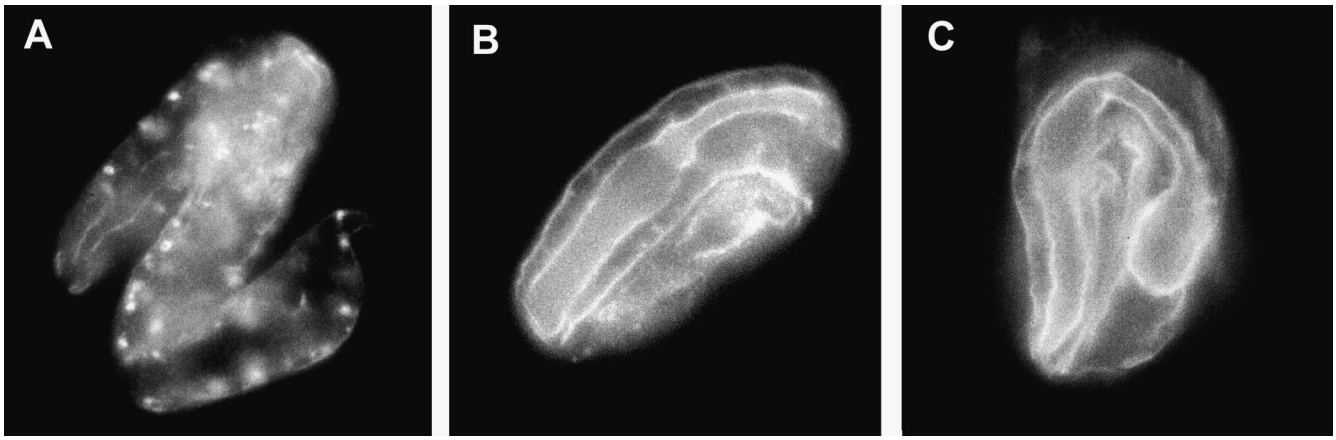


*Figure 7.* Type IV collagen distribution in a temperature-sensitive *let-2* glycine substitution mutant. Staining of the temperature-sensitive glycine substitution mutant *let-2(b246).* Embryos grown at 25°C (*A*), 20°C (*B*), or 15°C (*C*) were stained with LET-2 antiserum. Staining with EMB-9 antiserum gives an identical pattern. At 25°C, type IV collagen is seen as intense intracellular spots in body wall muscle cells. Very faint staining of basement membranes is seen in some embryos. At 15 and 20°C, type IV collagen is distributed in an essentially wild-type pattern.

*Table II. Distribution of the LET-2 Type IV Collagen Chain in let-2 Mutants**

| Strain | Percentage punctate intracellular stain‡ | | | Percentage pharyngeal basement membrane stain§ | | |
|---|---|---|---|---|---|---|
| | 15°C | 20°C | 25°C | 15°C | 20°C | 25°C |
| N2 | 3 | 0 | 10 | 100 | 87 | 87 |
| *unc-3(e151)* | 0 | 0 | 0 | 100 | 100 | 100 |
| *let-2(g25)* | ND | 15 | 73 | ND | 97 | 21 |
| *let-2(b246)* | 0 | 0 | 79 | 97 | 100 | 24 |
| *let-2(mn139)* | ND | 57 | 85 | ND | 55 | 9 |
| *let-2(mn109)* | 48 | 45 | 93 | 100 | 68 | 2 |
| *let-2(g30)* | 0 | 100 | 94 | 91 | 24 | 1 |
| *let-2(mn129)* | ND | 60 | 83 | ND | 83 | 33 |

*Embryos from homozygous mutant or wild-type (N2) mothers were stained with LET-2 and UNC-54 antisera. Only embryos that showed UNC-54 stain were examined. At least 30 embryos were scored for each strain at each temperature.
‡The percentage of twofold stage or later embryos that showed strong intracellular LET-2 stain in body wall muscle cells.
§The percentage of twofold stage or later embryos that showed strong LET-2 stain of the pharyngeal basement membrane.

high temperature, gaps in the body wall muscle quadrants can first be seen at the 1 1/2-fold stage and become extensive by the twofold stage (Fig. 10 *B*). In *emb-9* null mutant embryos, gaps also begin to appear at the 1 1/2-fold stage, although they are generally less numerous and less severe than in missense mutants. In two- to threefold *emb-9* null mutant embryos, numerous gaps in the body wall muscle quadrants are apparent (Fig. 10 *C*). Since gaps in the body wall muscle quadrants are seen in the *emb-9* null mutants, disruption of the body wall muscle quadrants can result from the absence of type IV collagen in the basement membrane. The first appearance of gaps in the body wall muscle quadrants corresponds to the beginning of muscle contractile activity, suggesting that the forces of contraction cause muscle cells to detach from the body wall.

## Discussion

In wild-type and *emb-9* or *let-2* missense mutants, the EMB-9 and LET-2 chains always colocalize. In *emb-9* null mutants, the LET-2 chain is retained intracellularly, indicating that it requires EMB-9 to be assembled and/or secreted. All characterized type IV collagen molecules are heterotrimers containing two α1-like and one α2-like chains (Hudson et al., 1993). EMB-9 and LET-2 are α1 and α2(IV)-like chains and are likely to be the only type IV collagen chains in *C. elegans* (Guo and Kramer, 1989). If EMB-9 and LET-2 assemble into a single collagen molecule, then mutations in either would interfere with the folding of the triple-stranded collagen molecule, resulting in interference with the assembly and secretion of both chains. All of these considerations suggest that they assemble into a single type IV collagen molecule containing two EMB-9 and one LET-2 chains.

The mutations of the α1(IV) collagen gene *emb-9* show a range of phenotypic severities, similar to that found for the α2(IV) gene *let-2* (Sibley et al., 1994). Most mutations are substitutions for glycine residues in the Gly-X-Y repeat domain of the molecule. It was noted that the phenotypic severities of glycine substitution mutations in *let-2* are inversely correlated with the calculated helical stability of type IV collagen at the position of the mutation (Sibley

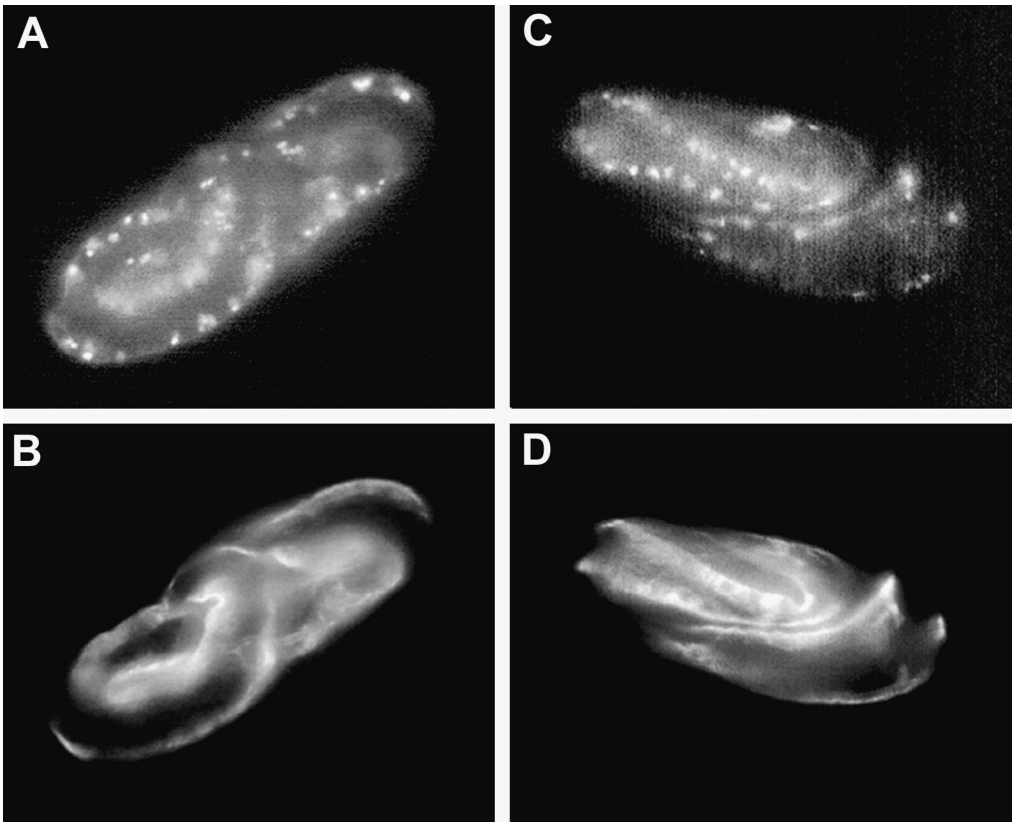


*Figure 8.* Type IV collagen distribution in a non–temperature-sensitive glycine substitution mutant. *emb-9(cg56)* embryos grown at 15°C (*A* and *B*) or 25°C (*C* and *D*) were stained with antisera against LET-2 (*A* and *C*) and body wall muscle myosin (*B* and *D*). Strong type IV collagen stain is seen within body wall muscle cells, while very weak stain is seen in the basement membrane beneath the muscle cells. The staining pattern is not affected by temperature and appears identical using EMB-9 antisera.

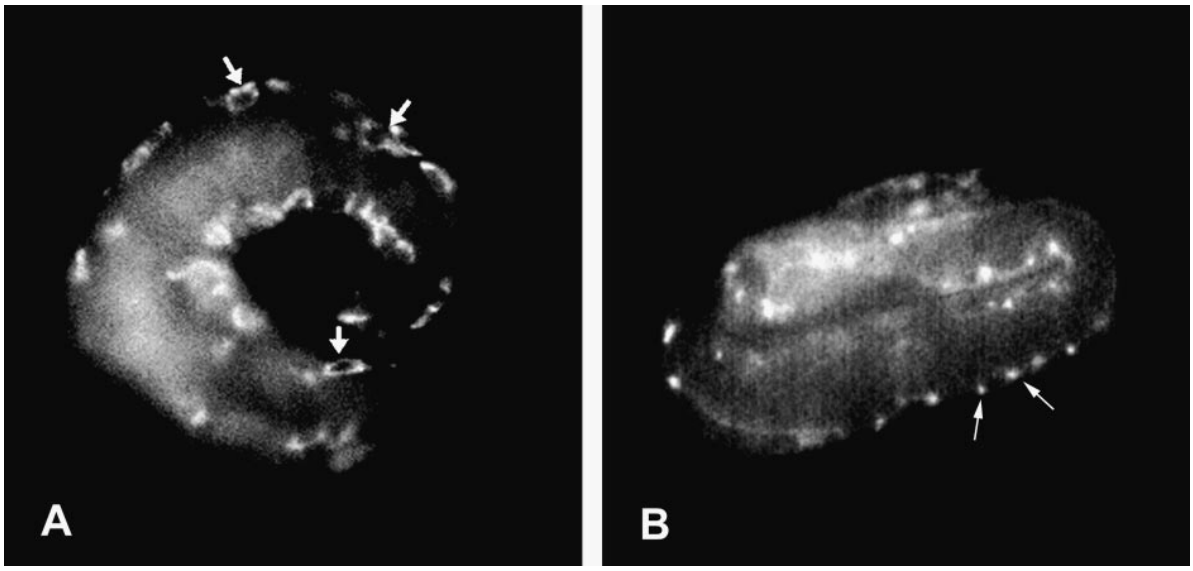


*Figure 9.* Type IV collagen distribution in Class II *emb-9* mutants. Staining of *st540* (*A*) and *cg56* (*B*) homozygous embryos with anti–LET-2 antiserum. Type IV collagen accumulates in a perinuclear ring in *st540* mutant embryos (*A*, *arrows*), while it primarily accumulates in two spots on either side of the nucleus in *cg56* mutant embryos (*B*, *arrows*). The same pattern is seen with EMB-9 and LET-2 antisera. *st545* shows the same pattern as *st540*, while all other *emb-9* and *let-2* mutants look like *cg56.*

et al., 1994). The most severe mutations are in regions of low stability, and the mildest mutations are in regions of high stability. This correlation is also apparent for the *emb-9* glycine substitution mutations. The mildest allele (*b117*) is in a region of above average stability, while the more severe alleles (*g23, hc70, b189, cg56*) are in regions of below average stability. For both *emb-9* and *let-2*, more severe alleles are located in lower stability regions, and milder alleles are in higher stability regions.

The phenotypes of most *emb-9* and *let-2* mutations are temperature-sensitive. As the temperature is increased, the percentage of arrested animals increases and arrest occurs earlier in development. Staining of mutant animals with type IV collagen antisera showed that as the temperature was increased, more of the mutant collagen was retained within cells, and less was detected in basement membranes. Thus, the more severe phenotype at higher temperature likely results because less type IV collagen is present in basement membranes. Mutant type IV collagen that is able to assemble into the basement membrane provides function and results in a less severe phenotype. We found no evidence for mutant type IV collagen that was able to assemble into basement membranes but did not provide function.

Assembly of collagen molecules requires registration of three chains at or near the carboxyl terminus, nucleation of the triple-helix, and propagation of the triple-helical structure towards the amino end of the molecule. The collagen triple helix is stabilized by hydrogen bonding and is an inherently temperature-sensitive structure (Engel and Prockop, 1991; Bachinger et al., 1993). Glycine residues are required at every third position because they are the only residue that can be accommodated in the center of the triple helix without distortion of the structure and loss

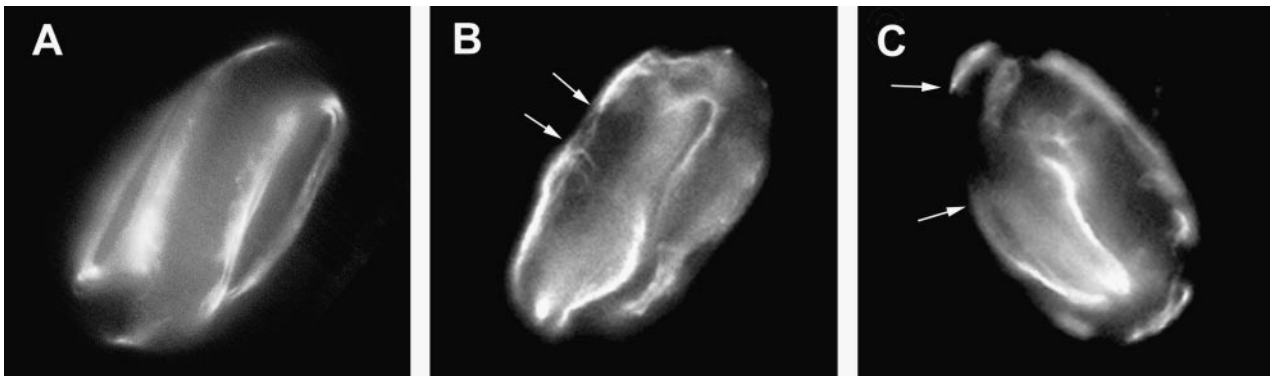


*Figure 10.* Body wall muscle disruption in *emb-9* mutants. Wild-type N2 (*A*), homozygous *g23* (*B*), and homozygous *cg46* (*C*) embryos were grown at 25°C and stained with antibody against body wall muscle myosin. In wild-type embryos, the muscle quadrants are continuous from head to tail. Gaps are seen in the muscle quadrants of the mutant embryos (*arrows*).

of stabilizing bonds. Glycine substitution mutations in the Gly-X-Y repeat domain of collagen interrupt the folding of the triple helix, requiring renucleation on the amino side of the substitution for triple helix assembly to progress.

*C. elegans* type IV collagen glycine substitution mutations can be viewed in terms of their effects on triple helix stability. Substitutions in regions of low helical stability cause more severe effects than those in high stability regions. Introduction of a glycine substitution in an already low stability region would produce such an unstable area that renucleation of triple helix assembly might be very inefficient or impossible. Milder alleles, located in more stable regions, would allow renucleation to occur more readily. The temperature sensitivity of most glycine substitutions could result because increased temperature would reduce the stability of the entire triple-helical domain. The mutations causing the most severe phenotypes are located in regions of low triple-helical stability, and a small increase in temperature would be sufficient to prevent assembly. The milder mutations are located in regions with higher stability, and larger increase in temperature would be required to completely block assembly.

The three putative null mutations generated in this study all have phenotypic characteristics distinct from other *emb-9* mutations. While all other mutations in *emb-9* and *let-2*, except *cg56*, cause arrest at approximately the twofold stage of embryogenesis, the null mutants arrest later, at the three- to fourfold stage. Since null mutant animals are capable of developing further than the missense mutants, the missense protein must interfere with the function of some other molecule(s). The missense chains act in a dominant negative fashion, interfering not only with the wild-type type IV collagen, but also with some other molecule(s). This interference could occur intracellularly, in the RER or Golgi network, and/or extracellularly, in basement membranes.

As shown here for *emb-9* and previously for *let-2* (Sibley et al., 1994), the dominant lethality of temperature-sensitive glycine substitution mutations increases with increasing temperature. Antibody staining shows that as the temperature increases, less type IV collagen is secreted. At the highest temperature tested, no type IV is detected in basement membranes. Since the dominance of these mutations becomes more severe as more type IV collagen is retained intracellularly and less is secreted, it is likely that dominant interference is occurring intracellularly, not in the basement membrane. Accumulation of large amounts of the abnormal collagen within cells could interfere with the synthesis, processing, and/or secretion of other proteins, thereby resulting in a more severe phenotype than results from simple loss of type IV collagen.

In *emb-9* null mutants, the LET-2 chain accumulates intracellularly but does not cause detectable dominant effects. Since LET-2 is the α2-like chain, it is not likely to undergo any trimeric assembly in the absence of EMB-9. In contrast, when there is a glycine substitution in either EMB-9 or LET-2, trimeric assembly is likely to initiate normally but be blocked when it reaches the site of the substitution. Intracellular accumulation of these partially assembled trimeric molecules appears to be more detrimental than accumulation of individual, unassembled LET-2 chains.

The dominant interference caused by mutations in type IV collagen and fibrillar collagens may result from different mechanisms. Glycine substitutions in mammalian fibrillar type I collagen inhibit collagen triple helix assembly and result in intracellular degradation of much of the mutant and normal collagen chains (Prockop et al., 1989; Engel and Prockop, 1991). Secretion of small amounts of glycine substitution containing type I collagen into the matrix can dominantly disrupt collagen fiber formation. Glycine substitutions can cause bulges or kinks in the collagen molecule (Prockop et al., 1989; Engel and Prockop, 1991). Since fibrillar collagens assemble into highly ordered, closely packed fibers, the presence of an abnormal collagen molecule distorts the packing of other molecules in the fiber. In contrast, type IV collagen normally contains 20 or more interruptions of its Gly-X-Y domain and forms a looser, less ordered network (Yurchenco and Ruben, 1987). Incorporation of a type IV collagen molecule containing a glycine substitution is not likely to greatly disrupt the type IV network. Collagen molecules containing glycine substitutions are likely to be much less disruptive to the type IV network than to the highly ordered fibers formed by fibrillar collagens. This conclusion is consistent with our evidence that the dominance of the *C. elegans* type IV collagen mutations is not an extracellular effect, but occurs intracellularly.

The effects on type IV collagen distribution in animals with mutations in type IV collagen genes appear somewhat different in mammals and *C. elegans.* In the kidneys of humans, mice, or dogs with mutations in the α3–α5(IV) collagen genes, type IV collagen is not detected in basement membranes or within cells (Gubler et al., 1995; Peissel et al., 1995; Cosgrove et al., 1996; Miner and Sanes, 1996; Thorner et al., 1996). In *C. elegans* mutants, type IV collagen can be reduced or absent from basement membranes but is correspondingly seen to accumulate within the cells that synthesize it. This difference could result from a higher synthesis rate and/or a lower degradation rate for type IV collagen in *C. elegans.* The *C. elegans* type IV collagen mutants arrest during embryogenesis, when there is high transcriptional activity of the type IV collagen genes (Graham et al., 1997), and the mutant animals are fixed and stained within a few hours after the time of their arrest. There may not be enough intracellular degradative activity in *C. elegans* to remove the accumulated type IV collagen during this short period.

In *emb-9* null mutants, we detected disruption of the body wall muscles beginning at the 1 1/2-fold stage, when contractions first begin. The disruption worsens as muscle contractions become more vigorous, and muscle cells appear to pull away from the hypodermis. It has been shown that body wall muscle function is required for elongation of the *C. elegans* embryo beyond the two-fold stage (Waterston, 1989; Barstead and Waterston, 1991; Williams and Waterston, 1994). The disruption of the body wall muscles that we see is sufficient to explain why the embryos arrest. At the time of arrest, the organization of other tissues, such as the pharynx and intestine, appears relatively normal. The type IV collagen that assembles into the basement membrane that separates body wall muscles from the hypodermis is critical for maintaining attachment of the body wall muscles during the force of contraction.

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