Mini-Collagens in Hydra Nematocytes

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Abstract. We have isolated and characterized four collagen-related c-DNA clones (N-COL 1, N-COL 2, N-COL 3, N-COL 4) that are highly expressed in developing nematocytes in hydra. All four c-DNAs as well as their corresponding transcripts are small in size (600–1,000 bp). The deduced amino acid sequences show that they contain a central region consisting of 14 to 16 Gly-X-Y triplets. This region is flanked amino-terminal by a stretch of 14–23 proline residues and carboxy-terminal by a stretch of 6–9 prolines. At the NH$_2$- and COOH-termini are repeated patterns of cysteine residues that are highly conserved between the molecules. A model is proposed which consists of a central stable collagen triple helix of 12–14 nm length from which three 9–22 nm long polyproline II type helices emerge at both ends. Disulfide linkage between cysteine-rich segments in these helices could lead to the formation of oligomeric network structures. Electrophoretic characterization of nematocyst extracts allows resolution of small proline-rich polypeptides that correspond in size to the cloned sequences.

NEMATOCYSTS are explosive organelles found in all species of cnidarians. They are mainly localized in tentacles and serve a variety of functions including the capture of prey and locomotion. Nematocyst capsules are a complex secretory product of the Golgi apparatus (Slautterback and Fawcett, 1959; Skaer, 1973; Holstein, 1981). More than 25 morphologically different capsule types have been described (Weill, 1934; Mariscal, 1974). They vary from 5 to 100 μm in size and consist of a stiff capsule wall enclosing a long intracapsular, armed tube. Following appropriate stimulation the capsule discharges and the tube is everted (Tardent and Holstein, 1982).

The explosive discharge of cnidarian nematocysts is one of the fastest and most spectacular events in biology. High speed cinematographical analysis has shown that the whole exocytotic process takes <3 ms (Holstein and Tardent, 1984). Components of the ejected tubule are accelerated to 40,000 g during the explosion (Holstein and Tardent, 1984). The very high osmotic pressure in resting capsules (Weber, 1989) and the extraordinary speed of tube evagination require that capsule wall and tube have a high tensile strength.

The molecular structure of nematocyst capsules is not understood. Early observations of hydroxyproline in capsules (Lenhoff et al., 1957) suggested that collagen-like molecules may be involved in the wall and the tube structure. However, such collagens appeared to be unusual since they seemed to be highly crosslinked by disulfide bridges (Blanquet and Lenhoff, 1966).

To gain a better understanding of nematocyst structure and differentiation we have isolated and sequenced nematocyte-specific transcripts from a cDNA library in hydra. One major class of transcripts encodes very small collagen-like peptides that have long polyproline stretches and a large number of cysteine residues. These unusual "mini-collagens" appear to be a major component of the inner wall of nematocyst capsules in hydra. The deduced amino acid sequences suggest a plausible structure consisting of triple helical collagen molecules which could be extensively crosslinked to each other.

Materials and Methods

Strains and Culture Conditions

For all our experiments we used strain sf-1 of *Hydra magnipapillata* (Sugiyama and Fujisawa, 1978). Strain sf1 has temperature-sensitive interstitial cells. Animals cultured at 18°C contain normal levels of interstitial cells and their derivatives, whereas animals cultured at 28°C lose their interstitial cells within 1–4 d. The animals were cultured in a modified hydra medium (Loomis and Lenhoff, 1956) at 18°C, fed daily with freshly hatched *Artemia nauplii* and washed 1 and 8 h later. Animals for experiments were selected from the culture 24 h after the last feeding.

Separation of Interstitial Cells from Epithelial Cells

Suspensions of dissociated cells were prepared by enzymatic digestion with pronase B from *Streptomyces griseus* (Serva, Heidelberg, FRG) at a concentration of 50 DMC-U ml^{-1} (in hydra dissociation medium; Gierer et al., 1972) and 5 × 10^8 Hydra cells/ml at 18°C for 4 h. Cells of the interstitial cell lineage, i.e., stem cells, nematocytes, gland, and nerve cells were enriched by sedimentation at 1 g for 15 min in a 15-cm column filled with 2 × 10^7 cells/ml. The top one-third of the suspension was removed and submitted to a second cycle of sedimentation. After the second cycle the top one-third of the suspension was significantly enriched in interstitial cells and their derivatives; it contained <1% epithelial cells.

Construction of an I-Cell cDNA Library from Hydra

Poly (A)+ RNA (6 μg per 5 × 10^6 cells from the I-cell–enriched fraction) was isolated by the method of Chirgwin et al. (1979) and used to construct

a cDNA library of 190,000 recombinant clones in lambda gt-10 as described by Huyhn et al. (1984).

Differential Screening of the I-Cell cDNA Library

8,000 recombinant clones of the cDNA library were differentially screened by the method of John and Davis (1979) at high stringency conditions (Singh and Jones, 1984). The two c-DNA probes (I-cell probe from an enriched I-cell fraction and epithelial probe from animals without I-cells) were prepared in the presence of ^{32}P-dCTP from poly A$^+$ RNA using MMLV-reverse Transcriptase (Berger and Kimmel, 1987). The concentration of the probes in the hybridization solution was 3×10^6 cpm/ml, respectively.

DNA Sequence Analysis

Commonly used recombinant techniques such as gel electrophoresis, subcloning, growth of plasmids and bacteriophage, and restriction nuclease digestions were carried out as described by Maniatis et al. (1982). For DNA sequence determination restriction fragments were cloned into a pUC 19 vector. DNA sequencing was done by the chain termination method (Sanger et al., 1977) using an USB sequencing kit from United Biochemical GmbH (Bad Homburg v.d.H., FRG) following the manufacturers instructions. Sequence data were analyzed with the sequence analysis software package, Hibio DnasisTM and Hibio ProsisTM from Hitachi Software Engineering Co., Ltd. (Yokohama, Japan).

Northern Analysis

Total RNA was extracted from whole polyps by the guanidinium isothiocyanate method (Chirgwin et al., 1979). Samples of total RNA were electrophoresed in a 1% agarose gel containing 2,2 M formaldehyde and transferred to a nylon membrane (Biodyne B from Pall GmbH, Dreieich, FRG). The RNA was hybridized and washed at high stringency conditions according to Singh and Jones (1984).

In Situ Hybridization to Mazerates

Mazerates were fixed in 4% formaldehyde as described (David, 1973). The cells were dehydrated in ethanol and prehybridized with a 10 μg/ml heparin solution for 5 min at room temperature. Probes were labeled with digoxygenin-dUTP using the random primer procedure according to the protocol of the Boehringer labeling kit. Hybridization was carried out in 100 ng/ml heparin, 100 mM DTT, 3× SSC, and 2 μg/ml denatured labeled probe in a humidified chamber at 65°C for 4 h. Washes were in 2× SSC at 65°C, 2× 20 min. The slides were routinely kept overnight at 4°C in 2× SSC. The hybridized probe was detected using the anti-Dig/AP detection kit from Boehringer Mannheim GmbH (Mannheim, FRG). Incubation in NBT/X-phosphate substrate was for 15–45 min. The cells were mounted with PBS/Glycerol (9:1) and analyzed using a Letiz Dialux microscope with 16×, 40× and 100× interference contrast optics (Kurz, 1991).

In Situ Hybridization to Whole Mounts

For fixation of whole mounts, polyps were anesthesized in 2% urethane and fixed with Lawdowsky's fixative (ethanol/formalin/acedic acid/water = 50:10:4:40) for at least 1 h. The polyps were then washed in PBT (PBS and 0.1% Tween 20) and incubated in PBT plus 50 μg/ml Proteinase K for 2 min. Proteinase K digestion was stopped by incubation for 2 min in 2 mg/ml glycine. After two washes in PBT (20 min) the polyps were postfixed in 4% paraformaldehyde and prehybridized in a solution containing 50% formamide, 5× SSC, 50 μg/ml heparin, and 0.1% Tween 20 for 1 h at 45°C. After adding the heat-denatured, digoxigenin-labeled probe (0.5 μg/ml), samples were hybridized for 9 h at 45°C followed by several washes in PBT with decreasing parts of hybridization solution. The hybridized probe was detected as described for macerates. Incubation in NBT/X-phosphate substrate was for 1–5 min. After staining the polyps were mounted with PBS/Glycerol and analyzed using the same optics as described for macerates (Tautz and Pfeiffle, 1989).

Isolation of Nematocysts

Intact, undischarged nematocysts were isolated from whole hydra tissue by centrifugation of frozen and thawed hydra tissue through 50% Percoll (in H$_2$O dest) at 10,000 rpm and 4°C using a HB4 rotor in a Kontron centrifuge.

Immunocytochemistry

Immunocytochemistry of isolated nematocyst capsules was performed according to Petri (1991). mAb H22 stains the outer wall of nematocyst capsules.

Pronase Treatment of Isolated Capsules

Isolated capsules were treated with Pronase B from Streptomyces griseus (Serva, Heidelberg) at a concentration of 10 DMC U ml^{-1} (in 0.1 M Tris, pH 7.5, 5 mM CaCl$_2$) for 2 h at 37°C.

PAGE

SDS-PAGE of solubilized nematocyst proteins was performed according to standard protocols (Hames and Rickwood, 1984) on 6–18% gradient or 12% gels using the Laemmli buffer system.

Labeling with [^{3}H]Proline and [^{3}H]Leucine and Autoradiography

Hydra were labeled with L-[2,3,4,5,-^{3}H]proline (3.70 TBq/mmol; NEN, Boston, MA) or L-[3,4,5,-^{3}H]leucine (5.18 TBq/mmol; NEN) by injecting the isotope into the gastric cavity using a polyethylene needle (David and Campbell, 1972). Animals were injected with 0.5 μl of the isotope at a concentration of 185 kBq/μl; nematocysts were isolated from 100 labeled hydra 4 d after labeling. For fluorography, protein gels were impregnated subsequently with [3H] enlightening scintillator (NEN), and 15% glycerol before drying and exposure (3 d).

Results

Isolation of Four Nematocyte-specific cDNA-Clones

Cells of the interstitial cell lineage (stem cells, proliferating nematoblasts, differentiating nematocytes, nerve cells, and gland cells) were separated from epithelial cells by differential sedimentation after dissociation of hydra tissue with pronase. The resulting preparation of cells contained only ∼1% epithelial cells compared to ∼20% in the initial suspension of dissociated cells. Poly A$^+$ RNA was isolated from the enriched I-cell fraction and used to establish a cDNA-library in lambda gt10. The library was differentially screened according to standard procedures (John and Davis, 1979) using ^{32}P-labeled cDNA probes prepared from I-cell poly A$^+$ RNA or from epithelial cell poly A$^+$ RNA. The epithelial cell RNA was prepared from a temperature-sensitive mutant strain (sf-1) that lacks all interstitial stem cells, nematoblasts, and nematocytes after a heat shock at 28°C for 4 d (Sugiyama and Fujisawa, 1977).

From a total of 8,000 clones, 600 were picked which hybridized more strongly with the I-cell probe than with the epithelial cell probe (Kurz, 1991). ^{32}P-labeled DNA prepared from one of the strongest differentially expressed clones (N-COL 1) crosshybridized to 100 of the 600 picked clones, indicating that these clones contained similar or identical sequences. Based on differences in the strength of crosshybridization, four clones (N-COL 1, N-COL 2, N-COL 3, N-COL 4) were presumed to represent distinct transcripts containing homologous regions. Subsequent sequence analysis (see below) confirmed this assumption.

To determine the cell type expressing the transcripts of the cloned sequences in situ hybridizations were carried out on macerated cells and fixed whole mounts with digoxigenin-labeled DNA. Hybridized sequences were identified with an antidigoxigenin antibody coupled to alkaline phosphatase.
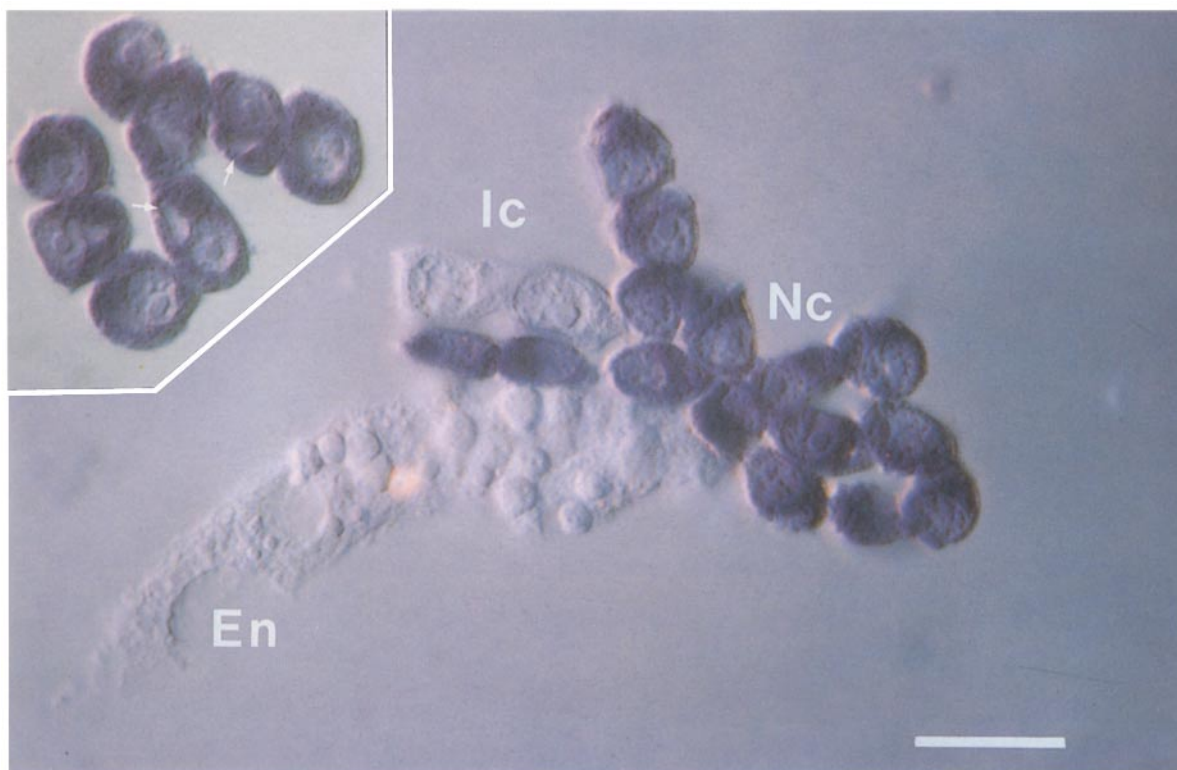
Figure 1. Expression of N-COL 1 RNA in mazerated cells. Early stages of differentiating nematocytes are heavily stained. Other cell types are unstained. Nc, nest of 16 differentiating nematocytes; Ic, interstitial stem cells; En, endodermal epithelial cells; arrows, developing nematocysts. Insert shows a nest of eight differentiating nematocytes with clearly identifiable vacuoles. Bar, 10 µm.

Fig. 1 shows macerated cells hybridized with clone N-COL 1. Nests of differentiating nematocytes are heavily stained. All other cell types were unstained even after prolonged incubation in the staining reaction. Closer analysis of the preparations indicated that all stained nematocytes contained developing capsules (Fig. 1, *inset*). Proliferating nematocyte precursors, which lack developing capsules, and mature nematocytes with fully differentiated nematocyst capsules were not stained. Thus, clone N-COL 1 expression is restricted to the postmitotic phase of nematocyte differentiation, during which capsule products are synthesized and the developing capsule grows in size.

Since N-COL 1 is only expressed in early stages before formation of mature capsules, it was not possible to determine whether a specific nematocyte type was stained. However, since all early stage nematocytes are stained we conclude that the N-COL 1 transcript or its cross-hybridizing homologues are expressed in all nematocyte types.

In whole mounts of hydra, N-COL 1 hybridized to clusters of cells throughout the gastric region and distal peduncle (Fig. 2). Such clusters have been shown to contain differentiating nematocytes by histological techniques (David and Challoner, 1974). Thus, the whole mounts confirm the localization of N-COL 1 expression in differentiating nematocytes. Furthermore, they indicate that expression of N-COL 1 occurs in a spatial pattern along the body column. Nematocytes expressing N-COL 1 were abundant throughout the gastric region but absent from the head and tentacle region and the proximal peduncle and basal disk.

Clones N-COL 2 and N-COL 3 gave identical staining pat-

terns to N-COL 1 after in situ hybridization (data not shown). Thus, it appears that these clones represent a family of transcripts that are strongly expressed in differentiating nematocytes.

Sequence Analysis of Nematocyte-specific Clones

The restriction sites and fragments used for sequencing the four clones are shown in Fig. 3. Fig. 4 shows the nucleotide sequences and the deduced amino acid sequences. N-COL 1 and N-COL 3 contain clearly defined open reading frames encoding peptides with 149 and 186 amino acids. Both have putative Met start codons followed by hydrophobic domains that have the characteristic features of signal peptides (von Heijne, 1983). N-COL 2 is incomplete at the NH2-terminus. By virtue of its close homology to N-COL 1, it appears to lack only the Met start codon and the first few amino acids of the leader sequence. N-COL 4 is also incomplete but closely homologous to N-COL 3 (see Fig. 5). It appears to lack 10–12 amino acids at the NH2-terminus including the anticipated leader sequence. A stretch of 154 nucleotides at the 5′ end of N-COL 4 does not show any homology with N-COL 3 or with the other clones. It does not contain a Met codon but encodes several stop codons in all three reading frames. This 5′ stretch does not appear to be part of the N-COL 4 coding region but represents an unknown DNA piece that has been accidently linked to the N-COL 4 DNA during preparation of the cDNA Library.

All four cDNA clones encode peptides with highly related structures (Fig. 5). The sequences have a central region containing 14–16 Gly-X-Y repeats typically found in collagens.
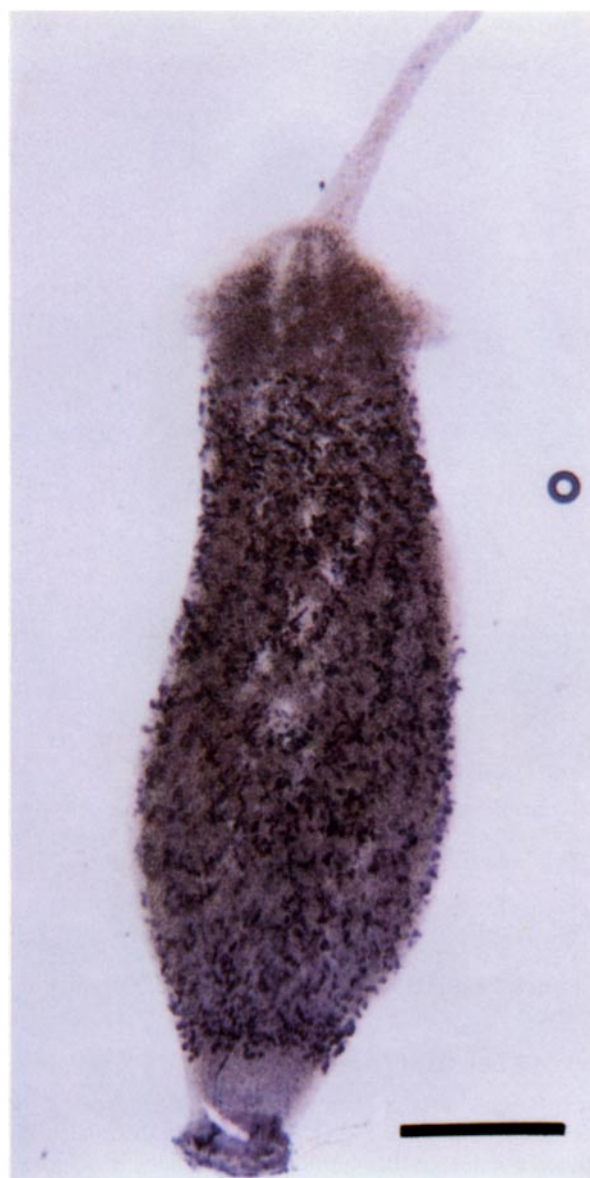
Figure 3. Structures of N-COL cDNAs with the restriction sites used for sequencing. Arrows show the strategy used for DNA sequence analysis. Clone-specific fragments that were subcloned for further experiments (see below) are indicated by hatched boxes. P, PvuII; S, SphI; E, EcoRI; R, RsaI; A, AluI; H, HaeIII.

Figure 2. In whole mounts clone N-COL 1 hybridizes to clusters of nematocytes throughout the gastric region and distal peduncle. No staining is observed in the head and tentacle region or in the proximal peduncle and basal disk. Bar, 500 μm.

These regions are flanked by long polyproline stretches. At the NH₂ and COOH termini there are well-defined repeat motifs with cysteine in every fifth position. Because of their small size we refer to the peptides as "mini-collagens."

Three of the four clones encode strongly hydrophobic domains at the NH₂-terminus that have the characteristic features of signal peptides (von Heijne, 1983). All four peptides contain a well-defined signal sequence processing site with alanin or serin in the −1 position relative to the processing site (Fig. 5). The NH₂-terminal sequences of the processed peptides are strongly hydrophilic; in all four cases the first amino acid of the proposed mature peptide is a lysine. Clone N-COL 4 is incomplete. However, its sequence is closely similar to clone N-COL 3. By comparison with clone N-COL 3 it is clear that clone N-COL 4 lacks only the sequence encoding part of the signal peptide.
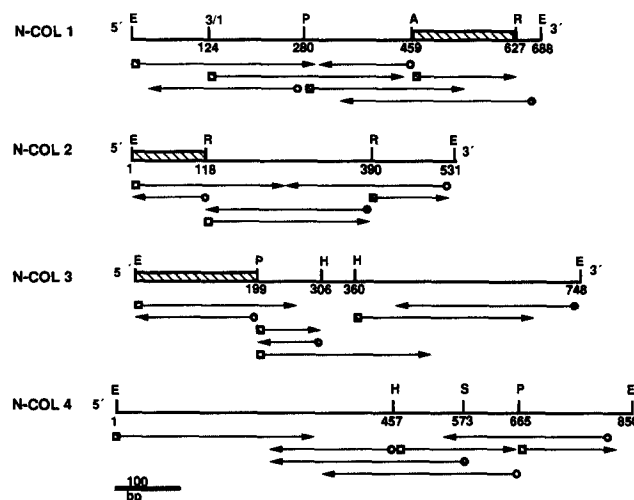
Although all four peptides have the same basic structure, they clearly fall into two groups in which the member peptides are more closely related to each other than to members of the other group (Fig. 5). Clones N-COL 1 and N-COL 2 encode peptides with 130 amino acids (excluding the leader sequence). They both have a central region with 14 Gly-X-Y repeats preceded by a stretch of 23 proline residues and followed by a proline-rich stretch of 8 residues. At both NH₂- and COOH-termini there are four identical repeat motifs with cysteine in every fifth position.

Clones N-COL 3 and N-COL 4 form the second group of closely related sequences. They encode peptides of 168 and 169 amino acids (excluding the leader sequence), respectively. Both peptides have a central region with 16 Gly-X-Y repeats. NH₂-terminal to this region is a stretch of 12 proline residues in clone N-COL 3 and 14 proline residues in clone N-COL 4. Directly following the Gly-X-Y sequence is a proline-rich stretch of 8 amino acids and a second long stretch of 12 prolines in clone N-COL 3 and 14 prolines in clone N-COL 4 near the COOH-terminus. Although several cysteine residues are present near the NH₂-terminus, there is no clear repeat pattern similar to that found in clones N-COL 1 and N-COL 2. There is, however, an isolated cysteine residue preceding the polyproline stretch similar to that found in clones N-COL 1 and N-COL 2. In both N-COL 3 and N-COL 4 there is a repeat structure consisting of four cysteine residues near the COOH-terminus and a second repeat of three Cysteine near the central Gly-X-Y region. This latter repeat is also present in clones N-COL 1 and N-COL 2.

Expression of Mini-collagen Sequences in Hydra Cells

Because of their high homologies in the proline and Gly-X-Y regions the four clones crosshybridize to each other (see above). Fig. 6 *a* shows that clone N-Col 1 hybridizes strongly to transcripts between 600–1,000 nucleotides long. It also hybridizes weakly to several larger transcripts. Because of the intensity and width of the 600–1,000 band, it appears to

1162

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                              cgaaagattcata
                    20
ATG GCT ATG CGA CTT GTC TTG GCA TGC CTC GTT TTA GGG GTG GCC GCT
Met Ala Met Arg Leu Val Leu Ala Cys Leu Val Leu Gly Val Ala Ala
            60                          80
ACT CAG GCC AAA ACT TTA CAC GAA ATG TTG AAA CGT GAT GCC AAT CCA
Thr Gln Ala Lys Thr Leu His Glu Met Leu Lys Arg Asp Ala Asn Pro
         100                         120                    140
TGC GGT AGT TAT TGC CCA TCT GTA TGT GCT CCT GCT TGT GCA CCA GTT
Cys Gly Ser Tyr Cys Pro Ser Val Cys Ala Pro Ala Cys Ala Pro Val
                     160                         180
TGT TGT TAT CCA CCA CCT CCT CCA CCA CCC CCA CCC CCA CCA CCA CCA
Cys Cys Tyr Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro
                200                         220                238
CCA CCA CCA CCA CCT CCA CCA CCA CCA CCA GCA CCA CTT CCA GGT AAT
Pro Pro Pro Pro Pro Pro Pro Pro Pro Ala Pro Leu Pro Gly Asn
                          260                         280
CCA GGA CCT CCA GGA CGA CCA GGA CCA CCA GGA GCA CCA GGA CCA GCT
Pro Gly Pro Pro Gly Arg Pro Gly Pro Pro Gly Ala Pro Gly Pro Ala
                     300                         320
GGA CCA CCA GGA CTT CCA GGA CCT CCA GGA CCA CCA GGA GCA CCA GGA
Gly Pro Pro Gly Leu Pro Gly Pro Pro Gly Pro Pro Gly Ala Pro Gly
            340                         360                    380
CAA GGA GGA CTT CCA GGA CAA CCT GCA CCA CCA CCA CCA CCC TGC CCA
Gln Gly Gly Leu Pro Gly Gln Pro Ala Pro Pro Pro Pro Pro Cys Pro
                     400                         420
CCA GTT TGC GTT GCT CAA TGT GTT CCA ACA TGC CCA CAA TAC TGT TGT
Pro Val Cys Val Ala Gln Cys Val Pro Thr Cys Pro Gln Tyr Cys Cys
                440
CCC GCA AAG AGA AAG taaactattcattttagctagcacattttctaattcaaatata
Pro Ala Lys Arg Lys ***

ttcctgactcagacgttttgaaaaaatgtccacgatagcaattgaagcgattcaaagtagacc
acgggcggagagattttgtttattgatgaaatggtagaagagatacgaaatgaaatatatta
tacatacatataatgtacaacgttaaactaatatcattttctttaataaaaaaaaaaaaaaaa
aaaa
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                    20                          40
GCA GCT TTC CTC CTC CTG GTT GCT GTT ACC AAC GCA AAA ACT CTG CAT
Ala Ala Phe Leu Leu Leu Val Ala Val Thr Asn Ala Lys Thr Leu His
            60                          80
GAA ACT TTA TCA AAG AGA AGT GCA CAA GCA TGT GGT TAT AAT TGT CCT
Glu Thr Leu Ser Lys Arg Ser Ala Gln Ala Cys Gly Tyr Asn Cys Pro
         100                         120                    140
GCT ATT TGT GCC CCA GCA TGT ACT CCA ATT TGC TGC GCT CCT CCT CCA
Ala Ile Cys Ala Pro Ala Cys Thr Pro Ile Cys Cys Ala Pro Pro Pro
                     160                         180
CCA CCT CCA CCA CCA CCT CCA CCA CCA CCA CCA CCA CCA CCA CCT CCA
Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro
                200                         220                238
CCA CCA CCA CCA GTT GCA ATT CCA GGA AAC CCA GGA CCA CCA GGA CGT
Pro Pro Pro Pro Val Ala Ile Pro Gly Asn Pro Gly Pro Pro Gly Arg
                          260                         280
CCA GGA CCT CCA GGA TTT CCA GGA CCA ATG GGA CCA CCA GGA CCC CCA
Pro Gly Pro Pro Gly Phe Pro Gly Pro Met Gly Pro Pro Gly Pro Pro
                     300                         320
GGA CCT CCA GGA CCA CCA GGT TAT CCA GGA GAA GGA GGA ATG CCA GGA
Gly Pro Pro Gly Pro Pro Gly Tyr Pro Gly Glu Gly Gly Met Pro Gly
            340                         360                    380
CAA CCT GCA CCA CCA CCA CCA CCA TGC CCA CCA ATC TGT CCA ACA CAG
Gln Pro Ala Pro Pro Pro Pro Pro Cys Pro Pro Ile Cys Pro Thr Gln
                     400                         420
TGT GTA CCA TAT TGC CCT CAG TAT TGT TGC CCA TTG AAA AAG taaaatg
Cys Val Pro Tyr Cys Pro Gln Tyr Cys Cys Pro Leu Lys Lys ***

gtcaagtttgtccagaatcaactttttattgaagaaagttgggtaaatgaataaaaatatgtaa
atatattagtttttgaaactataaaaaaaaaa
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                    20                          40
cg ATG GAT AAG AGG ATC CTT GTT CTC GTA TTT GCC GTT GTG GCT TAT
   Met Asp Lys Arg Ile Leu Val Leu Val Phe Ala Val Val Ala Tyr
            60                          80
GCC TCC GCT AAG TCC GTT GAT CTC GAA AAA AGA TCT CCA TGT GAT CCC
Ala Ser Ala Lys Ser Val Asp Leu Glu Lys Arg Ser Pro Cys Asp Pro
         100                         120                    139
GGC TGT GGC GCC GGA TGC GGT ATG GGT GGT ATG GGT GGA GCT GCT GGA
Gly Cys Gly Ala Gly Cys Gly Met Gly Gly Met Gly Gly Ala Ala Gly
                     160                         180
GGA TGC GGC GGA GCA GTT GTT CAA GAA ATT CCA ATG TCT ATA ATC AAT
Gly Cys Gly Gly Ala Val Val Gln Glu Ile Pro Met Ser Ile Ile Asn
                200                         220
CTT CCA GCT GGA CCA GCC CCA CCA TGT ATG CCA CCT CCC CCA CCC CCA
Leu Pro Ala Gly Pro Ala Pro Pro Cys Met Pro Pro Pro Pro Pro Pro
     240                         260                         280
CCA CCA CCA CCA CCA CCA TAC CCT GGT CCA CCC GGT GCT CCA GGA CCT
Pro Pro Pro Pro Pro Pro Tyr Pro Gly Pro Pro Gly Ala Pro Gly Pro
                          300                         320
ATG GGA CCA CCA GGA GGC CCA GGA TGC CCA GGT CCA CAA GGT CCA CCC
Met Gly Pro Pro Gly Gly Pro Gly Cys Pro Gly Pro Gln Gly Pro Pro
            340                         360                    379
GGA CCA CCA GGA GGC CCA GGA GGC CCA GGT ATG CCA GGA CCA CCA GGA
Gly Pro Pro Gly Gly Pro Gly Gly Pro Gly Met Pro Gly Pro Pro Gly
                     400                         420
CCA CCA GGA CCA CCA GGA ATA CCA GCC CCA CCA GCC CCA CCA CCA CCC
Pro Pro Gly Pro Pro Gly Ile Pro Ala Pro Pro Ala Pro Pro Pro Pro
                440                         460
CCA ATC TGT CTC CAT CAT TGT ATG AAA ATA TGC CCA GCT CCA GCA CCA
Pro Ile Cys Leu His His Cys Met Lys Ile Cys Pro Ala Pro Ala Pro
     480                         500                         520
GCA TGT GTA CCA CCA CCA CCT CCT CCA CCA CCA CCA CCA CCA CCA GCC
Ala Cys Val Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Ala
                540
TGC TCT TGT TAT CCA CCA CCA TGC TAC TGC GGT tagacagatctagttttcc
Cys Ser Cys Tyr Pro Pro Pro Cys Tyr Cys Gly ***

tttttacttaatgagtaacaacgaatgaagctgatacggaaagaactctcaaattttgctgtt
ttggatgtacgacaatagcctcagctacatatatatgtattttatatatatatctctttgac
taacaaaacaacaaatatatgatgtaaaattaaaaaaa
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                                     taaatgaaa
aataaaataaaaataatgaagatagtttgattttatttaattttattgaggtaatttcaatta
aaaccaacccaaacaacatctttgttctaaatttttatattgttattacaaatagttaaaaaa
                    20
aatccaaccctcaaatcaa ACT CCT TCC AAA AAT CCT CTA TTT TGT CGT TTA
                    Thr Pro Ser Lys Asn Pro Leu Phe Cys Arg Leu
     40                          60                         80
GAA AAA AGG TCT CCC TGT GGA TGT CAG GGC GGA TGC GGT GGA GGA GTT
Glu Lys Arg Ser Pro Cys Gly Cys Gln Gly Gly Cys Gly Gly Gly Val
                     100                         120
GGT GTT CAA GAA ATT CCA ATG ACA GTT ATC AGT ATT CCA TCT GGA GGA
Gly Val Gln Glu Ile Pro Met Thr Val Ile Ser Ile Pro Ser Gly Gly
            140                         160
GGA GGG GGC TAT GGT TGT GGA CCA CCA CCT CCT CCC CCT CCC CCT
Gly Gly Gly Tyr Gly Cys Gly Pro Pro Pro Pro Pro Pro Pro Pro
     180                         200                         220
CCC CCA CCT CCC CCA ATT CCC GGA CCA CCT GGA CCA CCA GGA CCA ATG
Pro Pro Pro Pro Pro Ile Pro Gly Pro Pro Gly Pro Pro Gly Pro Met
                          240                         260
GGA CCA CCA GGA CCT AAT GGT TAT GAT GGA CAG CAA GGA CCA CCA GGA
Gly Pro Pro Gly Pro Asn Gly Tyr Asp Gly Gln Gln Gly Pro Pro Gly
            280                         300                    319
CCT CCC GGA TGG AAT GGA CCT AAT GGG CCT CAA GGA CCA CCT GGA CCT
Pro Pro Gly Trp Asn Gly Pro Asn Gly Pro Gln Gly Pro Pro Gly Pro
                     340                         360
CCT GGC CCT CCT GGT ATC CCA GCC CCA CCT GCA CCA CCT GCA CCA CCA
Pro Gly Pro Pro Gly Ile Pro Ala Pro Pro Ala Pro Pro Ala Pro Pro
                380                         400
CTA TGT TTG CAC CAT TGT ATA AGA ATT TGC CCA GCA CCA TCC CCA GCA
Leu Cys Leu His His Cys Ile Arg Ile Cys Pro Ala Pro Ser Pro Ala
     420                         440                         460
TGC GTA GGT TTA CCA CCA CCC CCA CCC CCA CCA CCT CCA CCA CCC CCA
Cys Val Gly Leu Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro
                480                         500
CCA CCA TCA CCA ATG AGT TGT TTT TGC CAA CCC CAA CCT TGC AGC TGT
Pro Pro Ser Pro Met Ser Cys Phe Cys Gln Pro Gln Pro Cys Ser Cys

Gly ***
GGG taaaaatgaagtagttgtaaataaaagaaatatgttgaaactttttattttatatttaaa
aaatgattttgtataaaaatattatttaacacgattttaaaacttgtgttgcattttgtttta
cgaaaaagaatattaattttc(aaa)n
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Figure 4. Nucleotide and deduced amino acid sequences of the nematocyte-specific cDNA clones N-COL 1, 2, 3, and 4. Potential triple helical regions, which contain glycine as every third amino acid, are indicated by double lines. Polyproline regions are underlined. Cysteines are indicated in bold letters.

contain more than one transcript. This family of cross hybridizing transcripts presumably corresponds to the set of crosshybridizing mini-collagen clones which we have sequenced.

To confirm this hypothesis we prepared clone-specific probes from clones N-COL 1, N-COL 2, and N-COL 3. The specific probe for N-COL 1 was prepared using a PvuII, AluI restriction fragment from the 3' untranslated region (hatched box in Fig. 3). Hybridization of a Northern blot with this probe revealed an ~900 nucleotide transcript (Fig.

6 b). This probe did not recognize the other transcripts which are recognized by the complete N-COL 1 probe. Similar clone-specific probes prepared from clones N-COL 3 and N-COL 2 (Fig. 3) revealed transcripts of ~650 and 900 nucleotides, respectively. All three clone-specific probes did not cross hybridize to each other (see below) or to other I-cell specific transcripts (Fig. 6, lanes T). Clone N-COL 1 did however hybridize weakly with a transcript in the epithelial RNA (Fig. 6, lane E).
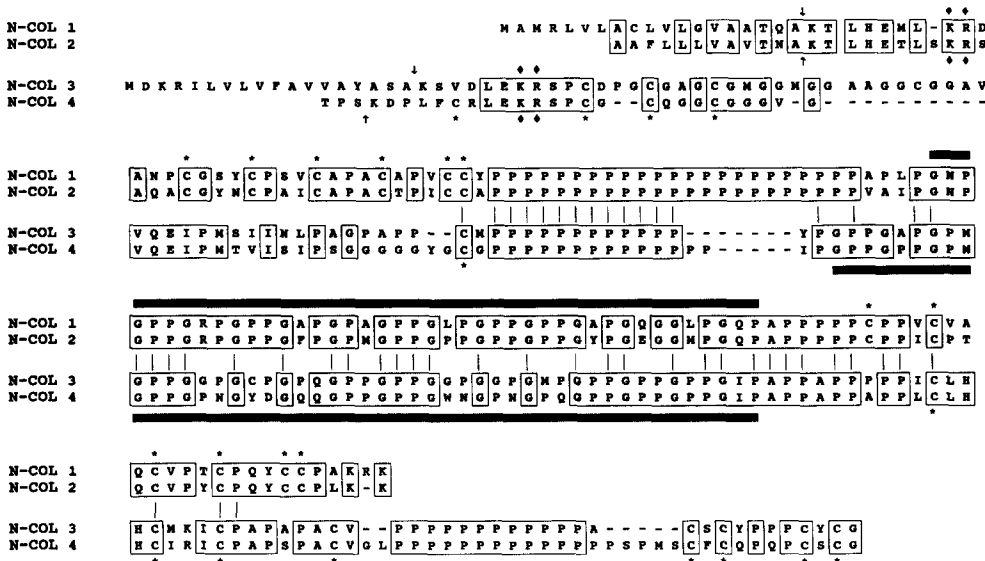
Using the clone-specific probes we determined the num-

N-COL 1 MAMRLVLACLVLGVAATQAKTLHEML-KRD
N-COL 2 AAFLLLVAVTNAKTLHETLSKRS
N-COL 3 MDKRILVLVFAVVAYASAKSVDLEKRSPCDPGCGAGCGMGGMGGAAGGCGGAV
N-COL 4 TPSKDPLFCRLEKRSPCG--CQGGCGGGV-G-----------
N-COL 1 ANPCGSYCPSVCAPACAPVCCYPPPPPPPPPPPPPPPPPPPPPPPAPLPGMP
N-COL 2 AQACGYNCPAICAPACTPICCAPPPPPPPPPPPPPPPPPPPPPPPVAIPGNP
N-COL 3 VQEIPMSIINLPAGPAPP--CMPPPPPPPPPPPP--------YPGPPGAPGPM
N-COL 4 VQEIPMTVISIPSGGGGGYGCGPPPPPPPPPPPPPP------IPGPPGPPGPM
N-COL 1 GPPGRPGPPGAPGPAGPPGLPGPPGPPGAPGQGGLPGQPAPPPPPCPPVCVA
N-COL 2 GPPGRPGPPGFPGPMGPPGPPGPPGPPGYPGEGGMPGQPAPPPPPCPPICPT
N-COL 3 GPPGGPGCPGPQGPPGPPGGPGGPGMPGPPGPPGPPGIPAPPAPPPPPICLH
N-COL 4 GPPGPNGYDGQQGPPGPPGWNGPNGPQGPPGPPGPPGIPAPPAPPAPPLCLH
N-COL 1 QCVPTCPQYCCPAKRK
N-COL 2 QCVPYCPQYCCPLK-K
N-COL 3 HCMKICPAPAPACV--PPPPPPPPPPPPA-----CSCYPPPCYCG
N-COL 4 HCIRICPAPSPACVGLPPPPPPPPPPPPPPSPMSCFCQPQPCSCG

Figure 5. Amino acid similarity among the putative translation products of N-COL 1, 2, 3, and 4. Amino acid homologies between group N-COL 1, N-COL 2 and group N-COL 3, N-COL 4 are boxed. Identities between the two groups are indicated by vertical lines. Gly-X-Y regions are emphasized with fat horizontal lines. Asterisks indicate repeating cysteines. Arrows show the putative signal processing sites and filled diamond heads indicate two basic amino acids (*KR*) that can be found in all four clones 6–9 amino acids following the processing site.

ber of homologous clones in the original cDNA library. 12 and 13 clones, respectively, hybridized to the N-COL 1 and N-COL 2 specific probes. The clone N-COL 3 probe hybridized to six clones in the library. A clone-specific probe for clone N-COL 4 was not prepared. None of the clones cross-hybridized with each other although all of them cross-hybridized with probes prepared from the complete clone sequences. Thus, transcripts of all three clones occurred in the total mRNA population approximately at a frequency of 0.08–0.15%. Their frequency in single nematocytes, however, must be ∼0.5–1.0% since nematocyte mRNA constituted ∼16% of the mRNA which we initially used to prepare the cDNA probe and the I-cell library.

Ⓐ
- 28 S
- 18 S
Ⓑ
N-COL 1
N-Col 2
N-COL 3
T E
T E
T E
- 28 S
- 18 S

Figure 6. (*A*) Hybridization of N-COL 1 (complete cDNA) to 10 μg total RNA (from interstitial cells and epithelial cells). (*B*) Hybridization of clone specific fragments from N-COL 1, N-COL 2 and N-COL 3 which were prepared as described (Fig. 3). T, total RNA; E, RNA from epithelial cells (10 μg per lane). The filters were reprobed with ribosomal DNA to show that equal amounts of RNA were loaded (see lower part).

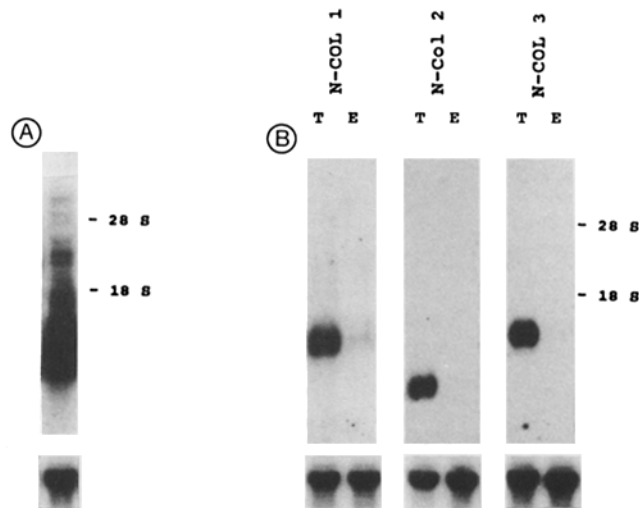

Are Single Mini-collagen Clones Expressed in Specific Nematocyte Types?

The occurrence of a family of highly related mini-collagen sequences suggests the possibility that these sequences are specifically expressed in single nematocyte types. To test this hypothesis we performed in situ hybridizations with clone-specific sequences. The results in Table I indicate that the same fraction of differentiating nematocytes hybridized to the total N-COL 1 probe as to the three clone-specific probes. Thus, the clone-specific sequences must be expressed in all types of differentiating nematocytes.

This conclusion was confirmed by close inspection of the morphology of hybridizing nematocytes following in situ hybridization. Only early differentiation stages, which lack clearly identifiable capsules, express the mini-collagen clones. Although such early stages are morphologically similar, they differ greatly in size. Large cells differentiate stenoteles; small cells differentiate desmonemes and isorhizas. Since the three clone-specific probes hybridized to all size classes of differentiating nematocytes, it is clear that each nematocyte type expresses all three mini-collagen sequences.

Identification of Mini-collagen Peptides in Nematocyte Capsules

The identification of mini-collagen genes expressed in differentiating nematocytes suggests that these peptides are

Table I. N-COL Expression in Differentiating Nematocytes

Clone	Stained nematocyte nests
	%
N-COL 1 (total)	75
N-COL 1 (specific)	77
N-COL 2 (specific)	77
N-COL 3 (specific)	70

Percentage of nematocyte clusters in macerates that were stained with clone-specific probes or with the N-COL 1 DNA. 100 clusters were counted per determination.

Downloaded from www.jcb.org on April 21, 2008
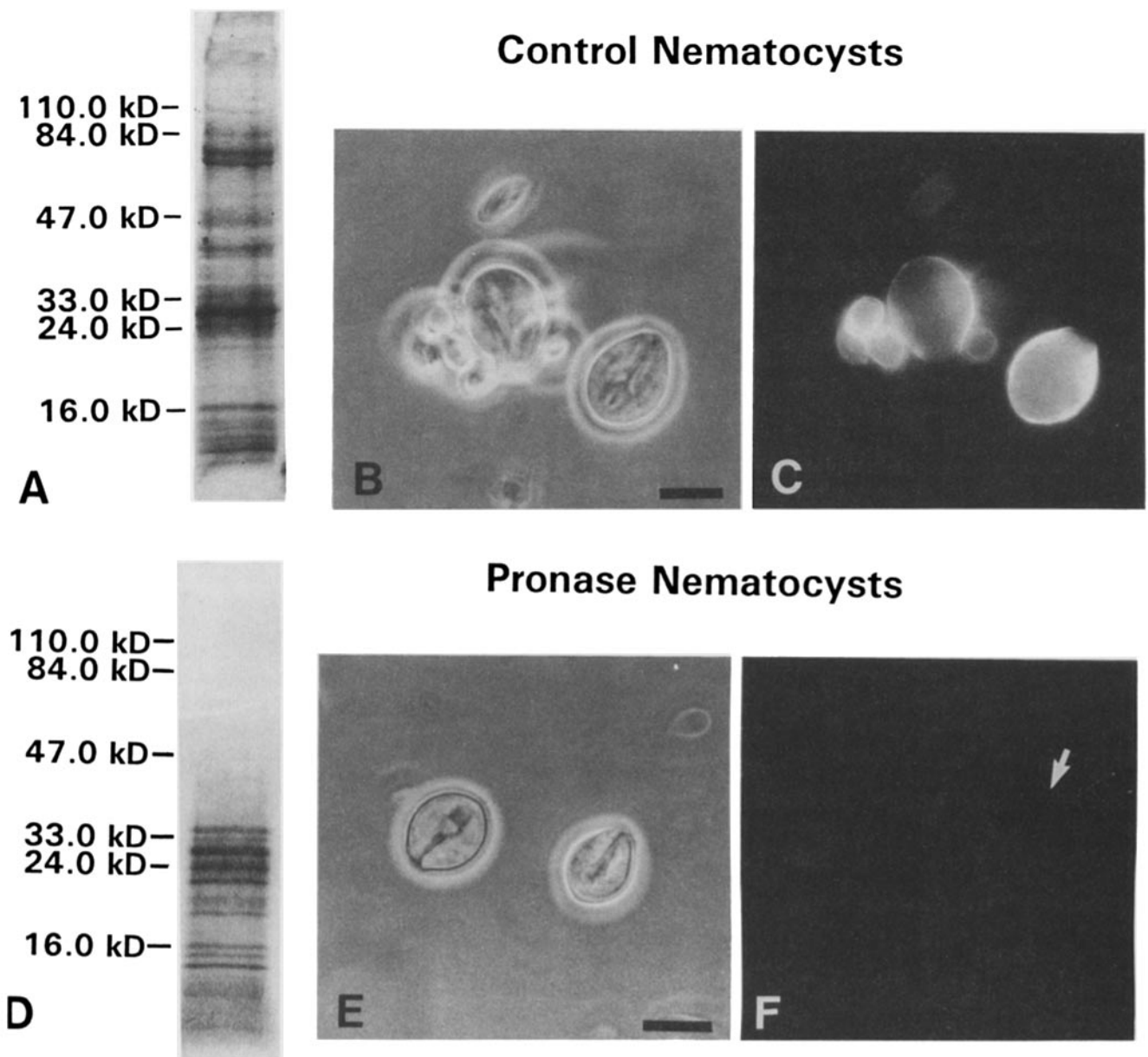
Control Nematocysts

110.0 kD—
84.0 kD—

47.0 kD—

33.0 kD—
24.0 kD—

16.0 kD—

A

B

C

Pronase Nematocysts

110.0 kD—
84.0 kD—

47.0 kD—

33.0 kD—
24.0 kD—

16.0 kD—

D

E

F

Figure 7. Digestion of the outer wall in isolated nematocysts using pronase B. (*A–C*) Control nematocysts; (*D* and *E*) pronase-treated nematocysts (2 h A and D protein pattern of isolated nematocysts in Coomassie-stained 6–18% gradient gels; *B, C, E,* and *F* demonstrate the effect of pronase treatment. (*B* and *E*) Phase-contrast micrographs; (*C* and *F*) micrographs of nematocysts stained with an outer wall specific mAb localized with indirect immunofluorescence (*FITC*). Bar, 10, μm.

constituents of nematocyst capsules. Capsules have been shown to contain large quantities of hydroxyproline (Lenhoff et al., 1957), and the capsule wall has been shown to contain cysteine crosslinked polymers which are insoluble in SDS but easily soluble in DTT (Blanquet and Lenhoff, 1966).

To identify mini-collagen peptides in mature nematocysts we isolated pure capsules from hydra by centrifugation of tissue homogenates through Percoll gradients. Under these conditions capsules form a pellet free of other cellular components. Such purified capsules have an outer wall and an inner wall, the latter being continuous with the tube that is coiled up inside mature capsules and which everts when capsules explode (Tardent and Holstein, 1982).

To analyze capsule proteins, purified capsules were dissolved in DTT and SDS and analyzed by PAGE. The result in Fig. 7 indicates a complex mixture of proteins varying

from 12 to 200 kD in size. The major proteins form size groups of 12–16, 30–40, 60–70, and 100–200 kD. This complex mixture could be markedly simplified by prior treatment of capsules with pronase (Fig. 7 *d*). Essentially all proteins larger than 40 kD were removed leaving only small proteins 12–16 and 25–40 kD in size.

Capsules are not dissolved by pronase treatment, nor do they explode. In phase-contrast microscopy they appear identical to untreated capsules (Fig. 7 *b*). They have however lost an outer wall specific antigen. Electron micrographs indicate that the outer wall has been removed by pronase treatment and that the inner wall is, at least morphologically, intact. Thus, it appears that the small capsule proteins remaining after pronase treatment constitute major components of the inner capsule wall.
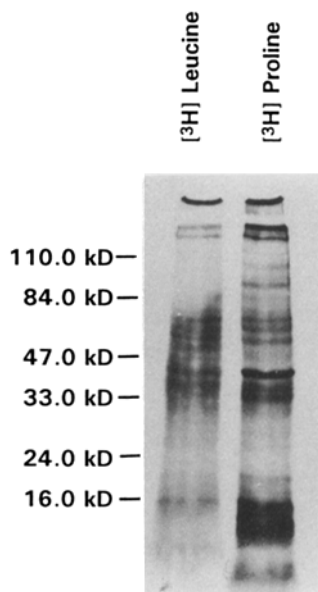
Based on their size the 12–16-kD proteins would appear

Figure 8. Autoradiogram of [³H]proline or [³H]leucine labeled nematocyst proteins (see Materials and Methods) run on a 6% PAA gel.

prediction can be made for the central region IV. Three chains must be connected in a collagen triple helix 12–14 nm in length to satisfy the hydrogen bonding capacity of the repeating glycines and the steric restrictions imposed by proline or hydroxyproline (Ramachandran, 1967; Ramachandran and Ramakrishnan, 1976). The abundance of proline and frequent occurrence in both X and Y position indicates a very stable triple helical structure. Its stability would be further increased by the occurrence of four hydroxyproline in the Y position (Engel et al., 1977). Extensive proline hydroxylation is expected in view of the high hydroxyproline content of the nematocyst capsules (Lenhoff et al., 1957).

Flanking the central triple helical domain are proline- or hydroxyproline-rich sequence regions that cannot participate in the triple helix because they lack glycine in every third position. These regions most likely assume the same polyproline II type structure which is found in the constituent helices of the collagen triple helix except that their mode of stabilization is different. Stretches of prolines in regions III, V, and VII are forced into polyproline II type structures by the steric restrictions imposed on the polypeptide backbone by proline rings and by hydration with water (Ganser et al., 1970). Stable isolated helices of this type have been observed in poly-L-amino acids and in proline and hydroxyproline rich plant (Cooper et al., 1987) and Volvox (Ertl et al., 1989) proteins. Since polyproline II helices have three amino acids per turn, this places cysteine residues in a threefold symmetry at the edges of putative polyproline II helices formed by the proline- and cysteine-rich regions II, VI, and VIII. Thus, disulfide bridging of the three chains already connected by the triple helical domains or between neighboring molecules is possi-
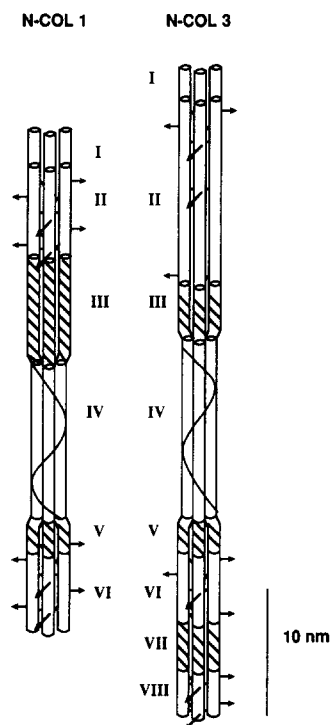
to be the peptide products of the mini-collagen clones. Since the deduced amino acid sequences (Fig. 4) suggest that the collagen peptides contain roughly 40% proline, we labeled hydra with [³H]proline, isolated mature capsules 4 d later, and analyzed capsule proteins by PAGE and autoradiography. The autoradiograph in Fig. 8 indicates that 12–16 and 30–40 kD proteins were more heavily labeled with proline than other components of capsules. By comparison, essentially all proteins in capsules were labeled with [³H]leucine. Thus, based on their size and their high content of proline, it appears likely that the 12–16-kD peptides in capsules are the products of the mini-collagen transcripts. Whether the 30–40-kD proline-rich peptides are dimers of the 12–16-kD peptides or the product of a larger transcript is presently unclear.

Discussion

Our results demonstrate the presence of transcripts encoding unusually small collagen peptides in differentiating nematocytes of hydra. The transcripts constitute a family of related sequences, of which four have been sequenced. The four sequences fall into two classes encoding mature peptides of 130 and 168/169 amino acids. Both classes have Gly-X-Y repeats in the center that can form a collagen triple helix structure. Because of this we refer to our peptides as mini-collagens.

Depending on type, collagen molecules consist of homo- or heterotrimers. From our sequence data it is not possible to predict directly the peptide composition of the putative collagen molecules. Potentially, the N-COL 1–N-COL 2 class of peptides could form two different homotrimers or a heterotrimer. Similarly, N-COL 3 and N-COL 4 could form homo- or heterotrimers. Because of their size differences it appears unlikely that mixed heterotrimers containing peptides from both classes would form.

The model in Fig. 9 is based on characteristic sequence features of distinct regions in the N-COL 1 and N-COL 3 polypeptide chains (designated by roman numbers) and on analogies with protein domains of known structure. A clear

Figure 9. Putative structure of two nematocyte-specific mini-collagens as derived from the primary sequences of N-COL 1 and N-COL 3. Different regions of the peptide sequences are indicated by roman numbers. Three peptide chains form a triple helix in region IV which has glycine in every third position (*curved line*). Stretches of proline form polyproline II helices which are arranged in parallel bundles (*III, V,* and *VII, hatched*). Cysteine-rich regions most likely also assume a poly proline II helical structure (*II, VI,* and *VIII*); in this structure cysteines are placed such that they can form S-S bridges between three peptide chains within one molecule (*bars*) or participate in intermolecular crosslinking (*arrows*). Region I starts at the first amino acid (lysine) of the mature peptide and contains a putative propeptide processing site. The sizes of the molecules were calculated assuming that the rise per residue in the triple helix is 0.29 nm and in the polyproline helix 0.31 nm.
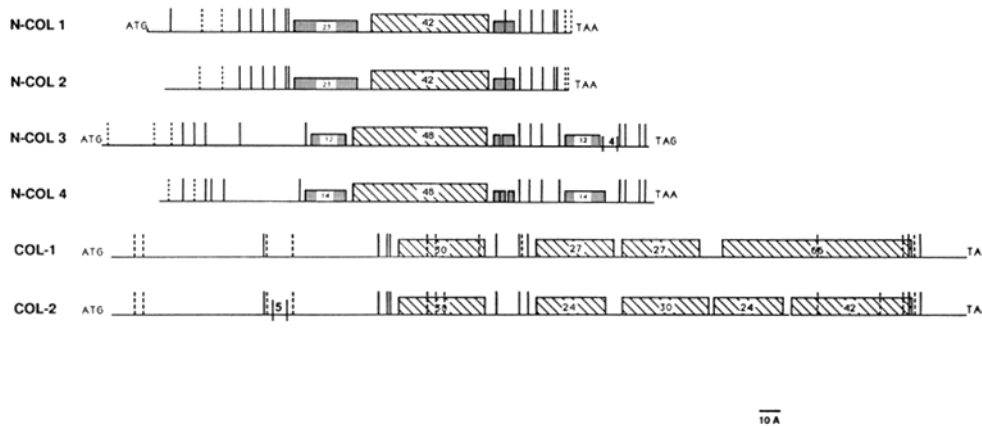
Figure 10. Structural comparison of the nematocyte collagens with cuticula collagens from *C. elegans* (*Col-1*, *Col-2*) (Kramer et al., 1982). The boxed, hatched areas represent triple-helical regions. Boxes that frame the polyproline regions are shaded. Nonhelical regions are represented by horizontal lines. Cysteine residues are indicated by solid vertical lines, and lysine residues by dashed vertical lines. For optimal homology, four amino acids were inserted into the N-COL 3 sequence and five amino acids were looped out in the Col-2 sequence.

ble (see Fig. 9). Stabilization by disulfide bridging has been demonstrated for two parallel polyproline II helices in the hinge regions of IgG 1 (Marquart et al., 1980) and IgG 3 (Johnson et al., 1975) and predicted for the terminal regions of component 8c-1 of α-keratin (Dowling et al., 1986).

Cysteines in regions II, VI, and VIII could lead to S-S bridged oligomeric structures consisting of many mini-collagen molecules. Alternatives to the models shown in Fig. 9 are also possible. The polyproline II helices in domains III, V, and VII may not necessarily be arranged in bundles but could emerge from the central domain IV in different directions giving rise to disulfide triple helices. Disulfide linkage may be between parallel chains as in the hinge regions of IgG or between antiparallel chains as in the NH₂-terminal regions of the network forming collagen IV (Martin et al., 1988). Details of the mode of assembly of the mini-collagens have to be explored by further work, but our results already indicate a disulfide-linked oligomeric network structure which is probably of major importance for the morphology and mechanical strength of the nematocyte capsules. The observation that capsule walls are only soluble under reducing conditions (DTT) supports this hypothesis.

Nematocyte Differentiation and the Synthesis of Mini-collagens

Nematocyst capsules are differentiation products of nematocytes. After a terminal mitosis, differentiating nematocytes commence capsule synthesis. Capsules are formed in vacuoles which grow in size by continuing fusion of Golgi-derived vesicles (Slautterback and Fawcett, 1959; Holstein, 1981). Thus, nematocyte proteins must be synthesized via ER and Golgi. Our results indicate that the mini-collagens have signal sequences required for synthesis in the ER. The peptides could also be posttranslationally modified, e.g., glycosylated, either in the ER or Golgi. However, none of the mini-collagen sequences have typical N-glycosylation sites (Asn-X-Ser) nor do they have lysine residues in the Gly-X-Y region which are often hydroxylated and glycosylated in collagens. This might result from the fact that nematocyte mini-collagens, unlike other collagens, are essentially intracellular molecules since they remain in the vacuole until the nematocyst is triggered to explode.

All four clones have Lys-Arg sequences 8–11 amino acids from the NH₂-terminus after removal of the putative leader peptide. Since endoproteases recognize and cut proteins at this sequence, it appears that the amino acids at the NH₂-terminus of mini-collagens may act as prosequences. This hypothesis is strengthened by the observation that the prosequences are strongly conserved in the two classes of mini-collagens. It is possible that such prosequences are involved in sorting of the mini-collagens to the developing nematocyst vacuole, in assembly of triple helical collagen molecules from peptides, or in controlling the formation of S-S bridges late in nematocyst development in association with hardening of the capsule wall (Günzl, 1968; Watson and Mariscal, 1984).

Mini-collagens and Capsule Morphology

Mutations in the collagen genes sqt (Kramer et al., 1988) and dpy 13 (von Mende et al., 1988) in the nematode *C. elegans* have been shown to alter cuticle morphology, indicating that these collagens are involved in defining morphological features.

Our results indicate that the different types of mini-collagens are not associated with specific types of nematocyst capsules. Three of the four sequences are expressed in all types of differentiating nematocytes; for the fourth sequence data are not available. Thus, capsule morphology is not simply related to a particular type of mini-collagens.

Comparison of Mini-collagens with Other Collagens

Collagens have been isolated and characterized from a number of coelenterates (Nordwig et al., 1973; D'Alessio et al., 1989; Kimura et al., 1983). Most of these collagens appear to be similar to large vertebrate collagens. However, there is no sequence data and only indirect information on the types of collagen involved. Partial DNA sequence data for sponge collagens indicate that these organisms have typical Type I and IV collagens (Exposito and Garrone, 1990; Exposito et al., 1990). All these collagens are significantly larger than the mini-collagens found in nematocytes.

Comparison of the hydra mini-collagen nucleotide sequences with published sequences in the EMBL genebank indicated 55–61% homology to *C. elegans* collagen genes

COL-1 and 48–55% homology to COL-2 (Kramer et al., 1982). The homology is primarily restricted to the Gly-X-Y encoding regions. Although the proline content of the Gly-X-Y regions in nematode collagens is high (31–36%) compared to other collagens, it is much lower than the 43–48% typical of nematocyte mini-collagens.

The nematode collagens are about twice as long as the nematocyte mini-collagens; however, both are much smaller than typical vertebrate collagens. The Gly-X-Y sequences in COL-1 and COL-2 occur in blocks interrupted by nonhelical regions. These blocks consist of 6–22 Gly-X-Y triplets, which is quite similar to the single Gly-X-Y block in the nematocyte mini-collagens (Fig. 10). COL-1 and COL-2 are rich in cysteine but they do not contain regular cysteine motifs as they are found in nematocyte collagens.

The unusual nature of mini-collagens parallels the unique nature of nematocyte capsules. Capsules are structurally and probably also evolutionarily related to extrusive organelles found in several classes of protozoa (Hausmann, 1978). Some of these organelles in myxosporidia, dinoflagellates, and ciliates exhibit tube discharge mechanisms similar to nematocysts. Molecular similarities between these structures have not yet been investigated. However, based on our results it appears possible that mini-collagen-like molecules occur in these protozoan structures.

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