Sequence and Transmembrane Topology of MEC-4, an Ion Channel Subunit Required for Mechanotransduction in *Caenorhabditis elegans*

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Abstract. The process by which mechanical stimuli are converted into cellular responses is poorly understood, in part because key molecules in this mode of signal transduction, the mechanically gated ion channels, have eluded cloning efforts. The *Caenorhabditis elegans mec-4* gene encodes a subunit of a candidate mechanosensitive ion channel that plays a critical role in touch reception. Comparative sequence analysis of *C. elegans* and *Caenorhabditis briggsae mec-4* genes was used to initiate molecular studies that establish MEC-4 as a 768–amino acid protein that includes two hydrophobic domains theoretically capable of spanning a lipid bilayer. Immunoprecipitation of in vitro translated *mec-4* protein with domain-specific anti–MEC-4 antibodies and in vivo characterization of a series of *mec-4lacZ* fusion proteins both support the hypothesis that MEC-4 crosses the membrane twice. The MEC-4 amino- and carboxy-terminal domains are situated in the cytoplasm and a large domain, which includes three Cys-rich regions, is extracellular. Definition of transmembrane topology defines regions that might interact with the extracellular matrix or cytoskeleton to mediate mechanical signaling.

MECHANOTRANSDUCTION, the conversion of a mechanical stimulus into a cellular response, plays fundamental roles in cell volume regulation, fertilization, gravitaxis, proprioception, and the senses of hearing, touch, and balance (for reviews see French, 1992; Sackin, 1995). Electrophysiological analyses have established that specialized ion channels mediate cellular responses to mechanical stimuli. Genes encoding components of mechanically gated channels, however, have proven difficult to isolate, and consequently, the molecular mechanisms of mechanotransduction are poorly understood.

The nematode *Caenorhabditis elegans* has been a fruitful system for the cellular and molecular study of mechanotransduction. Six mechanosensory neurons, called touch receptor neurons, sense gentle touch along most of the body (Chalfie and Sulston, 1981). Two genes, *mec-4* and *mec-10*, so designated because mutations in these genes render animals mechanosensory abnormal, encode members of a newly identified class of ion channel (Driscoll and Chalfie, 1991; Chalfie et al., 1993; Huang and Chalfie, 1994). Specifically, *mec-4* and *mec-10* proteins are related to subunits of the mammalian amiloride-sensitive epithelial Na^+ channel (ENaC) (Canessa et al., 1993, 1994*a*; Linguegila et al., 1993; Voilley et al., 1994; McDonald et al., 1994, 1995), which plays a central role in Na^+ reabsorption in distal kidney, colon, and lung (for review see Palmer, 1992). Because *mec-4* and *mec-10* are required for the function of the touch receptor neurons, are expressed nearly exclusively in these cells (Mitani et al., 1993; Huang and Chalfie, 1994), and encode proteins related to amiloride-sensitive ion channels (amiloride is a general inhibitor of mechanosensory ion channels in diverse organisms [Hamill et al., 1992]), MEC-4 and MEC-10 are candidate subunits of a mechanosensory ion channel.

A prerequisite for deducing the molecular mechanisms of action of this recently identified ion channel type is the determination of the primary structure of each subunit and their topologies with respect to the membrane. We have previously reported a partial coding sequence for MEC-4 that includes two major Cys-rich domains and a potential membrane-spanning domain (Driscoll and Chalfie, 1991). Here we report molecular studies that define the amino-terminal coding sequences of MEC-4 and establish that MEC-4 includes two transmembrane domains. Our biochemical and molecular genetic experiments show that the NH_2 and COOH termini of MEC-4 project inside the

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1. *Abbreviations used in this paper*: CAP, cyclase-associated protein; DAPI, 4′,6-diamidino-2-phenylindole; endo H, endoglycosidase H; GST, glutathione-*S*-transferase; lf, loss-of-function; RT, reverse transcription.

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cell, while a large central domain, which includes the Cys-rich regions, is situated outside the cell. Since mechanical gating is thought to require application of tension to a channel domain by a component of the extracellular matrix, the cytoskeleton or the membrane itself (e.g., see Hudspeth, 1989; Hamill and McBride, 1992; Sukharev et al., 1994), establishing subunit topology defines target regions for channel contacts likely to be essential for mechanotransduction.

Materials and Methods

Isolation and Sequence Analysis of C. briggsae mec-4

Radiolabeled *C. elegans mec-4* sequences were used to identify related sequences in a *C. briggsae* genomic library provided by D. Baillie (University of British Columbia). A 2.9-kb EcoRI fragment proved to include most *mec-4* coding sequences. An adjacent 6-kb HindIII fragment was partially sequenced to generate data for the 3′ end of *C. briggsae mec-4*; 0.35- and 0.5-kb EcoRI fragments were subcloned to generate sequence data for the 5′ end of the gene. Double-stranded DNA from a series of successive deletion subclones (Erase-a-base system; Promega, Madison, WI) was sequenced on both strands according to manufacturer's specifications (Sequenase; U.S. Biochemical Corp., Cleveland, OH); some regions were sequenced with specific oligonucleotide primers.

mec-4 cDNA Isolation and Characterization

Total RNA for reverse transcription (RT)[1]/PCR was isolated from wild-type *C. elegans* strain N2 using the TRIzol reagent (GIBCO BRL, Gaithersburg, MD). PolyA(+) RNA was purified using PolyATtract beads (Promega). Reverse transcription was as described (Stratagene, La Jolla, CA), using primer CCTGGATCCGTTACATGGGACCTGCTCGAATACT, which spans the junction of *mec-4* exons 8 and 9. For PCR amplification, antisense primer ATGGCCCTTCGCAGTTTTGTTATC, which spans the junction of exons 5 and 6, or TCGAATTTTGTTCTCTCTGATTGAC, which spans the junction of exons 4 and 5, was used in conjunction with sense primer AATGTCATGGATGCAAAACCTGAA, which includes the likely initiation codon. The PCR product was cloned into vector PCRII (Invitrogen, San Diego, CA) and sequenced to verify the integrity of the coding region. The longest *mec-4* cDNA isolated from a library was from the *C. elegans* λGT11 library described in Okkema and Fire (1994).

Plasmid Constructions

ZB#37 *mec-4*Δ-385 and ZB#38 *mec-4*Δ266, which harbor deletions of *mec-4* nucleotides −595 to −385 and −595 to +266, respectively (numbered as in Fig. 2 *A*), were constructed from the genomic *mec-4* clone TU#12 (Driscoll and Chalfie, 1991) using Exonuclease III (Erase-a-base; Promega). ZB#39 (*mec-4*M1L), ZB#40 (*mec-4*M4L), and ZB#41 (*mec-4*M20L), *mec-4* alleles that encode Leu in place of candidate initiation codons, and ZB#42 (*mec-4*Met1XX), which encodes two termination codons immediately after the *mec-4* AUG codon at position +28, were synthesized according to the method of Kunkel et al. (1987) (Mutagene; BioRad Laboratories, Hercules, CA), using the genomic *mec-4* clone TU#12 as a template.

The full-length *C. elegans mec-4* cDNA appears somewhat toxic in *Escherichia coli*, which complicated the cDNA work. To construct a full-length *mec-4* cDNA, introns 1–4 from the genomic *mec-4* clone TU#12 were first removed (Mutagene; BioRad Laboratories) to make clone ZB#43. Oligonucleotides used for looping out introns were: intron 1, ACAGAATCAGAAGTACAATGTGAACTTATTACGGGA; intron 2, GATATTCAATTGAAATTTGACACTGCACCTTTTCCA; intron 3, CAAAAAGGTATTTAATATTAATGATGCGATGAGGAATGGG; intron 4, AGAATGTCAATCAGAGAGAACAAAATTCGACGAGCC. A 1.9-kb HindIII–PvuI fragment including joined exons from ZB#43 was ligated to TU#10 (Driscoll and Chalfie, 1991) to reconstitute a full-length *mec-4* cDNA, ZB#44. To position the full-length cDNA downstream of the T7 promoter, an SmaI site was introduced in front of the *mec-4* initiation codon by PCR amplification of sequences in ZB#44, and a 0.2-kb SmaI–EcoRI fragment encoding the amino terminus was ligated to the 82-bp EcoRI fragment, the 2.1-kb EcoRI–HindIII fragment, and vector Bluescript pKS(−) to generate ZB#45. ZB#46 is the same construct as ZB#45 except that it harbors the *mec-4(d)* cDNA encoding the death-inducing A713V substitution, which was introduced by site-directed mutagenesis. ZB#47 harbors an NH_2-terminal FLAG-tagged *mec-4* cDNA gene in pKS^- that was constructed by hybridizing two complementary single-stranded oligomers that encode the FLAG epitope (Immunex, Seattle, WA) and a heart muscle kinase recognition site flanked by SmaI and SacI sites and ligating the SmaI–SacI fragment into ZB#45.

mec-4lacZ fusion ZB#48 was constructed by amplifying *mec-4* promoter and genomic coding sequences from TU#12 using 5′ T3 primer and 3′ primer TTTTTCTAGAGCTTCACCAATCATGGG, digesting with HindIII and XbaI and ligating the resultant product into the *lacZ* plasmid pPD21.28 (Fire et al., 1990). This creates a hybrid gene encoding MEC-4 amino acids 1–102 fused to β-galactosidase. *mec-4lacZ* fusion ZB#49 was constructed by amplifying *mec-4* promoter and genomic coding sequences from TU#12 using 5′ T3 primer and 3′ primer TTTTTCTAGACGTGCCGGTTTTGTGGT, digesting with HindIII and XbaI and ligating into the *lacZ* plasmid pPD21.28 (Fire et al., 1990) to generate a hybrid gene encoding MEC-4 amino acids 1–214 fused to β-galactosidase. *mec-4lacZ* fusion ZB#50 was constructed by subcloning a 2.9-kb HindIII–XbaI fragment harboring the *mec-4* promoter and coding sequences for amino acids 1–370 (derived from TU#12) into HindIII–XbaI digested *lacZ* plasmid pPD22.04 (Fire et al., 1990).

Glutathione-*S*-transferase (GST)–*mec-4* fusion plasmid ZB#51 was constructed by introducing an SmaI site in front of the *mec-4* initiation codon using PCR (template plasmid ZB#45), and then subcloning the SmaI–EcoRI fragment encoding MEC-4 amino acids 1–69 into pGEX-3X (Pharmacia Fine Chemicals, Piscataway, NJ). GST–*mec-4* fusion plasmid ZB#52 was constructed by introducing an EcoRI site into a 0.5-kb PCR-amplified fragment (template plasmid ZB#45) that encoded MEC-4 amino acids 449–626 within an EcoRI–EcoRV fragment. This fragment was cloned into SmaI–EcoRI-digested pGEX-3X. GST–*mec-4* fusion plasmid ZB#53 was constructed by subcloning a 0.3-kb HincII fragment from plasmid ZB#45 that encoded MEC-4 amino acids 705–768 into SmaI-digested pGEX-3X.

Strain Maintenance and Nematode Transformation

Strains were grown at 20°C and were maintained as described in Brenner (1974). Transgenic lines were constructed by injecting plasmids at 50 μg/ml as described (Mello et al., 1992; Driscoll, 1995). All constructs tested were coinjected with pRF4, which encodes *rol-6(su1006)* (Kramer et al., 1990), and causes transformants to roll. Since transgenic animals are mosaic for the presence of transforming DNA, three independently derived lines that exhibited a high frequency of segregation of the introduced DNA array were always selected for assay of phenotype.

Antibodies

To raise AbM4(1-69), we induced expression of GST/MEC-4(1-69) from ZB#51 in *E. coli* and purified the fusion protein over glutathione agarose (Pharmacia Fine Chemicals) as described (Smith and Johnson, 1988). The GST/MEC-4(1-69) antigen was injected into rabbits by Rutgers Laboratory Animal Services. To raise AbM4(527-539) and AbM4(552-561), peptides PFPDTFGYSAPTGC and SRLPAPYGDC were synthesized, conjugated with keyhole limpet hemocyanin, and injected into rabbits (Multiple Peptide Systems, San Diego, CA). To raise AbM4(746-760), peptide CAEHNYSLYKKKKAEKA was synthesized, conjugated with keyhole limpet hemocyanin, and introduced into rabbits (Pocono Rabbit Farm).

Antibodies used in immunoprecipitation experiments were purified over protein A–agarose columns (Boehringer Mannheim Biochemicals, Indianapolis, IN). Columns were equilibrated with 100 mM Tris-HCl, pH 7.5, loaded with antisera, and washed extensively with equilibration buffer. IgGs were eluted in 100 mM glycine, pH 3.0, and neutralized by adding 1/10 vol of 1 M Tris-HCl, pH 7.5. Antibodies used for whole mount staining were purified first on protein A–agarose columns, and were subsequently affinity purified using appropriate GST–MEC-4 fusion proteins. To generate MEC-4 affinity resins, GST–*mec-4* fusions in plasmids ZB#51, ZB#52, and ZB#53 were expressed in *E. coli*, purified on glutathione agarose, and separated by SDS-PAGE. The proteins were electroblotted to nitrocellulose paper, visualized by staining with Ponceau-S (Sigma Chemical Co., St. Louis, MO), and excised on strips. Protein strips were incubated with blocking solution (1× PBS and 2% nonfat dry milk) for 1 h and then with the protein A–purified IgG fraction at room temperature for 1 h. The paper was washed several times with 1× PBS, and bound antibody was eluted with 200 μl per 3 cm^2 of 100 mM glycine buffer (pH 2.5) and neutralized with 1/10 vol of 1 M Tris-HCl, pH 7.5.

Whole Mount Histochemistry

Wild type and ZB2 (a strain harboring ~18 copies of an integrated array of TU#44, a *mec-4lacZ* fusion that encodes MEC-4 amino acids 1–760 [Mitani et al., 1993]) were stained with antibodies as described (Driscoll, 1995). Antibodies were added at a dilution of 1:300; secondary goat anti-rabbit antibodies were rhodamine conjugated. Monoclonal anti-β-galactosidase antibody was from Boehringer Mannheim Biochemicals. Animals were mounted in 90% glycerol, 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), 1 mg/ml of *p*-phenylenediamine. Transgenic animals bearing *lacZ* fusions were stained for β-galactosidase activity as described in Fire et al. (1990). Positively staining animals were always detected within 12 h of incubation with X-gal; negatively staining animals showed no evidence of β-galactosidase activity after 48 h.

In Vitro Transcription/Translation Experiments

Plasmid DNAs were purified by alkaline lysis and passed over a QIAGEN type-100 column to remove RNA. Plasmids were linearized, phenol extracted, precipitated, and resuspended in diethyl pyrocarbonate–treated H_2O at 1 μg/μl. 2.5 μg linearized plasmid was added to 40 μM ATP, 40 mM CTP, 40 mM GTP, 40 mM UTP, 5 U RNasin, 30 mM DTT, and 25 U T7 RNA polymerase in 1× transcription buffer (Stratagene manual), total volume 25 μl. After 90 min at 37°C, the reaction was terminated by adding 1 μg DNase and incubating at 37°C for 5 min. The cRNA was extracted twice with phenol-chloroform and once with chloroform, ethanol precipitated, and resuspended at ~1 μg/μl in diethyl pyrocarbonate–treated water. For translation, 1 μg cRNA was denatured at 95°C for 1 min and immediately put on ice. Translation in 25 μl total volume was with 1 μg cRNA, 40 μM amino acids minus methionine, 20 μCi [^{35}S]methionine, 5 U RNasin, and 17.5 μl rabbit reticulocyte lysate (Promega) at 30°C for 1 h. In some reactions, 1.8 μl canine pancreatic microsomal membranes (Promega) were added. Reactions were terminated on ice, and products were analyzed using SDS-PAGE.

For endoglycosidase H (endo H) digests, 12 μl of in vitro translation product was mixed with 12.8 μl 10% SDS, denatured at 80°C for 3 min, and returned to ice for 5 min. 48 μl of endo H reaction buffer (50 mM Na_2HPO_4, pH 6.85, 0.2% β-mercaptoethanol, 0.5 mM PMSF), 10 mU of endo H (Boehringer Mannheim Biochemicals), and PMSF (final concentration 1 mM) were added, and the final volume was adjusted to 96.8 μl with H_2O. Digestion was at 37°C for 3 h. To concentrate the sample, an equal volume of 20% TCA was added, and the sample was incubated on ice for 30 min. Protein was centrifuged at 13,000 rpm. The pellet was washed three times with 1 ml cold 80% acetone, resuspended in 10 μl 1× sample buffer, and analyzed by SDS-PAGE. For proteinase K digestion, 5 μl in vitro translation product was equilibrated with 10 mM Ca^{+2}, and proteinase K (Promega) was added to a final concentration of 40 μg/ml. After incubation on ice for 1 h, the reaction was terminated by adding PMSF to 1 mM. To test the requirement for microsomes for protease protection, we added Triton X-100 to 1% (vol/vol) before proteinase K treatment.

Immunoprecipitation

5 μl of in vitro translation product and 5 μl of protein A–purified antibodies were mixed in 300 μl IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 1 mM PMSF) and incubated at 4°C for 1 h with gentle rotation. 30 μl of a 50% slurry of protein A–agarose was added to the mixture, and tubes were rotated at 4°C for 30 min. Samples were spun at 12,000 rpm for 12 s at room temperature and washed with IP buffer three times. After adding 1× sample buffer and heating at 95°C for 30 s, samples were analyzed by SDS-PAGE.

Results

Definition of the Amino-terminal Coding Sequence of MEC-4

Previous analysis of genomic and partial cDNA sequences of *C. elegans mec-4* left the amino-terminal coding sequences undefined (Driscoll and Chalfie, 1991). To determine the sequence of the MEC-4 amino terminus, we compared *mec-4* genomic sequences from *C. elegans* and *C. briggsae*, two species believed to have diverged 20–50 million years ago (Emmons et al., 1989). Sequence comparisons have established that exons and some regulatory sequences are highly conserved between the two species, whereas introns are markedly diverged (Heine and Blumenthal, 1986; Zucker-Aprison and Blumenthal, 1989; Heschl and Baillie, 1990; Lee et al., 1992; Kuwabara and Shah, 1994). Coding regions are easily identified as highly conserved open reading frames that differ primarily at the third base position of codons.

The *C. briggsae mec-4* gene was cloned by screening a *C. briggsae* genomic library for clones hybridizing to *C. elegans mec-4* coding sequences. Sequence analysis of cross-

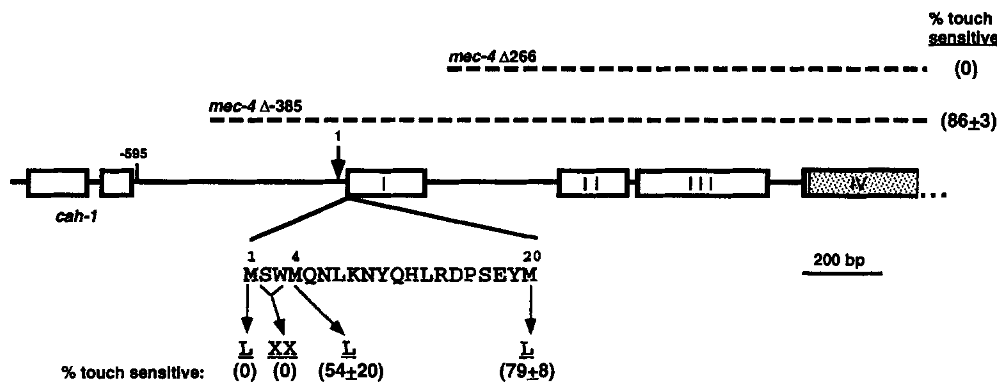

Figure 1. Organization of genomic sequence in the *mec-4* 5′ region and summary of mutational analyses that helped define MEC-4 NH_2-terminal sequences. Schematically diagrammed to scale are 2.3 kb of *C. elegans* genomic DNA upstream of previously identified *mec-4* coding sequences. White boxes represent newly identified open reading frames conserved between *C. elegans* and *C. briggsae*; black lines represent nonconserved intervening sequences; stippled box indicates a known *mec-4* exon (Driscoll and Chalfie, 1991). *cah-1* exons homologous to CAP protein are noted; *mec-4* exons are indicated by Roman numerals. The large arrow indicates the 5′ end of the longest *mec-4* cDNA cloned from a library, numbered nucleotide +1. Dashed overlines indicate sequences included in *mec-4* deletion derivatives Δ-385 and Δ266. The protein sequence highlighted indicates M1, M4, and M20, candidate NH_2-terminal MEC-4 residues. The percentage of *mec-4(lf)* mutants that are rescued by *mec-4* deletions Δ-385 or Δ266, by *mec-4* alleles encoding Leu substitutions for M1, M4, or M20, or by a *mec-4* allele that encodes two termination codons after M1 (M1XX) are indicated in parentheses. Scores are the average of at least 100 animals tested for each of three independently derived transgenic lines ± SD. Transgenic animals are mosaic for the presence of introduced DNA, and since all cells do not include the DNA array, rescued populations are not expected to include 100% touch-sensitive animals. Dominant *rol-6(su1006)* in plasmid pRF4 (Kramer et al., 1990), which was coinjected with all constructs tested to facilitate identification of transformants, does not affect the *mec-4(lf)* phenotype (0/300 transgenic animals).

A

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                                                                                                                   •••••••
-595 CAGTTCAAGACAACTTTTGTCAATGGCAAGCTAGTCACTGTCGTCTCGGACATTTGCTAAATCTTCTCACGTCATAACCTTATTTTTCAACTTCATGAACTATCCCCTCGAACTTTTTTA

                                    CRI                                                  CRII
-475 TTTGTCCCTGTCTACAATTCTTTATAAGCAATTGAGACACACTTTGCTTTATTAAATAATACCGATTTTGTTTTGTATATGCATTGACTGGTGCTCAACTATTTTTATTGAAAAAATTAC

                                                                                                                         •
-355 TTTTTACTGTCTCGTCGAATAAAAGTGATCCCTTCCAAAAAATAGTTATTCATGTTTGAAGGTTTGTGATCGTAGAACAATTGCACCGAATGTAGTGTAAAAAAAATTCCAGAATGTTCA

     ••••••                                                                                  CRIII
-235 AATACATTTCCACCCTCACCAGAACATCTCAAACACCAGGCACATTTCAACACACTTTCATGGATCTTATCTTGCCTCAAAACCATCAGCTTTCTTCTCATTCTACAAGTATGAAACAAT

           •••••  CRIV                                                                                                ♦
-115 CTACTATTCTATACATTTATAATGTAATGGAACCATGTGAAGACATGAGCAAGATCATTGTCGTCAGCGACTACCCCTCCTTCATTTTTCTTAGAGTATCAGTTTGCTAATGCTATTTTT
                                                      CRV

   6 TTTATCGCTATCAAGTTATAGAATGTCATGGATGCAAAACCTGAAAAACTACCAACACCTTCGGGACCCATCCGAGTACATGTCCCAGGTTTATGGAGACCCGTTAGCGTACTTACAAGA
                                 M  S  W  M  Q  N  L  K  N  Y  Q  H  L  R  D  P  S  E  Y  M  S  Q  V  Y  G  D  P  L  A  Y  L  Q  E   33
 126 GACGACTAAATTTGTGACAGAAAGAGAATATTATGAAGATTTTGGTTATGGCGAATGTTTCAACTCTACAGAATCAGAAGTACAAGTGAGTTTACGTAATTTTCAAATTGCAACGGTAAA
      T  T  K  F  V  T  E  R  E  Y  Y  E  D  F  G  Y  G  E  C  F  N  S  T  E  S  E  V  Q                                       61
 246 TTTTATTTTTGTATGCAATTGTATTTTGTTCCCAGTTTTTGAAATATTCGTTATATACATTGAAGTTTTTCAACTGTAAATTTGAAGGCCCGTATTTTCTTAATTCTTCGATAAGTATTT
 366 TGTCTTTATAACTACTAAGTTTTTGCTGAATCAACTTGTTTATTTTACTTTGATAGCTTATCTGCAAAACTGTACCAAAATTAAAAACCAAGATTTTTTGTTTTGGAACAATATAATCAA
 486 AACGCTTAAAATGTTCAATAAACGGTAAAAAAAATAAAACTTGCAAAAAGTCGCAGCTGAGTAATCTAACATTTCCGAATTTCAGTGTGAACTTATTACGGGAGAATTCGATCCAAAATT
                                                                                                  C  E  L  I  T  G  E  F  D  P  K  L   73
 606 GCTACCCTATGACAAACGTCTAGCCTGGCATTTCAAAGAGTTTTGCTACAAAACATCTGCTCACGGAATTCCCATGATTGGTGAAGCTCCAAACGTATATTATCGAGCAGTTTGGGTCGT
      L  P  Y  D  K  R  L  A  W  H  F  K  E  F  C  Y  K  T  S  A  H  G  I  P  M  I  G  E  A  P  N  V  Y  Y  R  A [V  W  V  V] 113
 726 ACTTTTTCTTGGATGTATGATAATGCTCTATCTTAATGCTCAATCTGTGCTTGACAAATACAACAGGAATGAGAAAATTGTCGATATTCAATTGAAATTTGGTAGTTTGAAAAATCATGT
     [L  F  L  G  C  M  I  M  L  Y  L  N  A  Q  S  V  L] D  K  Y  N  R  N  E  K  I  V  D  I  Q  L  K  F  D                   147
 846 GCTCCTTACTGAGCTTTTTTATTTCCAGACACTGCACCTTTTCCAGCAATTACGCTTTGTAATTTGAATCCTTACAAAGCAAGTTTAGCAACAAGCGTGGATTTAGTAAAGCGAACGTTG
                                         T  A  P  F  P  A  I  T  L  C  N  L  N  P  Y  K  A  S  L  A  T  S  V  D  L  V  K  R  T  L  177
 966 TCAGCATTTGATGGAGCAATGGGAAAAGCCGGAGGAAACAAAGATCACGAAGAGGAACGCGAAGTTGTCACCGAACCACCCACCACCCCTGCACCCACCACAAAACCGGCACGTCGTCGA
      S  A  F  D  G  A  M  G  K  A  G  G  N  K  D  H  E  E  E  R  E  V  V  T  E  P  P  T  T  P  A  P  T  T  K  P  A  R  R  R  217
1086 GGAAAACGTGATTTATCTGGAGCATTTTTTGAGCCAGGATTTGCAAGATGCCTTTGTGGAAGCCAAGGGTCTAGTGAGCAAGAGGATAAGGATGAGGAGAAGGAGGAAGAGTTACTTGAA
      G  K  R  D  L  S  G  A  F  F  E  P  G  F  A  R  C  L  C  G  S  Q  G  S  S  E  Q  E  D  K  D  E  E  K  E  E  E  L  L  E  257
1206 ACAACTACCAAAAAGGTATTTAATATTAATGGTGAGTGTTGCATGTCAAATGAGCATCGCGTAAAACATTCTTTTGAACACAGATGCCGATGAGGAATGGGATGGAATGGAAGAATATGA
      T  T  T  K  K  V  F  N  I  N  D                                                                A  D  E  E* W  D  G  M  E  E  Y  D  280
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Figure 2. *mec-4* gene structure and homology to the degenerin/epithelial Na^+ channel family. (*A*) The 5′ end genomic sequence of *C. elegans mec-4* and deduced amino-terminal coding sequences. Nucleotide numbers are indicated at the left; position 1 corresponds to the first residue of the longest *mec-4* cDNA isolated, which is indicated by a black diamond. Sequence included begins immediately 3′ to coding sequences of a CAP homologue situated adjacent to *mec-4.* Nucleotide stretches in the 5′ untranslated region conserved in *C. briggsae* and *C. elegans mec-4* genes, designated CRI-V, are underlined. Dotted overlines indicate sequences similar to the consensus binding site for the transcription factor UNC-86 (AAATTCAT; Xue et al., 1992), which binds to the *mec-3*, *mec-7*, and *mec-4* promoters (Xue et al., 1992, 1993; M. Chalfie, unpublished observations). A solid overline indicates a sequence similar to the consensus site for binding of MEC-3 (ATAATNNAT; Xue et al., 1992), a homeodomain protein required for expression of touch cell–specific genes, including *mec-4* (Way and Chalfie, 1988). Amino acid numbers are indicated on the right. The predicted first membrane-spanning domain is boxed in black. The asterisk marks E272, which is the first residue encoded by the partial *C. elegans mec-4* cDNA sequence (Driscoll and Chalfie, 1991). The sequence reported here includes an insertion of 82 bp after position 1587 in the genomic *mec-4* sequence previously reported. The sequence data for *C. elegans mec-4* and *C. briggsae mec-4* are available from EMBL/GenBank/DDBJ under accession numbers U53669 and U53670, respectively. (*B*) Alignment of *C. elegans* and *C. briggsae* MEC-4 with other *C. elegans* and mammalian Na^+ channel family members. The complete *C. elegans* MEC-4 sequence is listed; for *C. briggsae* MEC-4, only amino acids differing from *C. elegans* MEC-4 are indicated. Aligned also are *C. elegans* MEC-10 (Huang and Chalfie, 1994), DEG-1 (Shreffler et al., 1995; García-Añoveros et al., 1995), rat epithelial Na^+ channel, αrENaC (Canessa et al., 1993), βrENaC, and γrENaC (Canessa et al., 1994*a*). MEC-4 domains indicated are predicted α-helical membrane-spanning domains MSDI and MSDII (*black boxes*), Cys-rich domains CRDI, CRDII, and CRDIII (*grey boxes*), and a 22–amino acid region in DEG-1 implicated in channel closing that appears to play a similar role in MEC-4 (*white box*; García-Añoveros et al., 1995). Alignment was performed using the pileup program of the GCG sequence analysis package (Needleman and Wunsch, 1970). The consensus line highlights residues common to at least four family members.

hybridizing clones revealed five conserved open reading frames immediately 5′ to the start of the partial *C. elegans mec-4* cDNA clone previously characterized (schematically depicted in Fig. 1). The two most 5′ candidate exons correspond to the carboxy-terminal coding exons of the *C. elegans cah-1* gene (M. Chalfie, unpublished data), a gene that encodes a homologue of the cyclase-associated protein (CAP) (Field et al., 1990). Several observations support that the CAP-homologous exons do not encode part of the *mec-4* protein: (*a*) splicing signals that could ligate the CAP-homologous sequences in frame to the downstream *mec-4* exons are absent; (*b*) efforts at RT/PCR using 5′ primers corresponding to *cah-1* coding sequences and 3′ primers within known *mec-4* coding sequences were unsuccessful; and (*c*) a deletion clone lacking the CAP-homologous sequences and sequences 5′ to position −384 of the *C. elegans mec-4* sequence reported here (ZB#37 *mec-4*Δ-385) rescued the touch-insensitive phenotype of a loss-of-function (lf) *mec-4* mutant (Fig. 1). We conclude that CAP-homologous exons are not required for *mec-4* function, and that their presence reflects the syntenic organization of the *cah-1* and *mec-4* genes in *C. elegans* and *C. briggsae.*

Sequences upstream of and including the third exon identified are required for *mec-4* gene activity since a *mec-4* allele lacking these sequences (ZB#38 *mec-4*Δ266) failed to complement a *mec-4(lf)* mutation. The three downstream conserved exons proved to encode MEC-4 amino-terminal sequences: (*a*) we isolated a *mec-4* cDNA from a λGT11 library that includes the three predicted NH_2-terminal exons and begins 27 nucleotides upstream of the first in-frame ATG (Fig. 2 *A*); and (*b*) RT/PCR products amplified using 5′ primers from putative *mec-4* exon 1 and 3′ primers from the junctions of exons 4/5, 5/6, or 8/9 in-

B

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            1
MEC-4 C.e.  MSWMQNLKNY Q.......HL RDPSEYMS.. QVYGDPLAYL QETTKFVTER EYYEDFGYGE CFNSTESEVQ CELITG.... EFDPKLLPYD KRLAWHFKEF CYKTSAHGIP
MEC-4 C.b.  ---------- ---------- ---------- ---------- --N------- ---------- ----S----- ---------- ---------- ---------- ----------
MEC-10      MNRNPRMSKF QPNPRSRSRF QDETDLRSLR SFKTDFSNYL ASDTNFLNVA EIMTSYAYGE SNNAHEKEIQ CDLLTENGGI EIDPTRLSYR ERIRWHLQQF CYKTSSHGIP
DEG-1       .......... .......... .......... .........M SNHHSKTKKT SMLGREDYIY SHDITNKNKK EKLNGASKNN DYNQD..DDD ETMKSKMMDF CDKTTAHGAK
αrENaC      .......... ..MMLDHTRA PELNIDLDLH ASNSPKGSMK GNQFKEQDPC PPQPMQGLGK GDKREEQGLG PEPSAPRQPT EEEEALIEFH RSYRELFQFF CNNTTIHGAI
βrENaC      .......... .......... .......... .......... .......... ..MPVKKYLL KCLHRLQ.KG P.GYT..... .......... ..YKELLVWY CNNTNTHGPK
γrENaC      .......... .......... .......... .......... .......... .MAPGEKIKA KIKKNLPVRG PQAPT..... .......... ..IKDLMHWY CMNTNTHGCR
Consensus                                                                  Y                T                           F C  T  HG
            111                 MSDI
MEC-4 C.e.  MIGEAP.NVY YRAVWVVLFL GCMIMLYLNA QSVLDKYNRN EKIVDIQLKF DTAPFPAITL CNLNPYKASL ATSVDLVKRT LSAFDGAMGK AGGNKDHEEE REVVTEPPTT
MEC-4 C.b.  ---------- ------M--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----E-DG- K--I--A---
MEC-10      MLGQAP.NSL YRAAWVFLLL ICAIQFINQA VAVIQKYQKM DKITDIQLKF DTAPFPAITL CNLNPYKDSV IRSHDSISKI LGVFKSVMKK AG........ ..........
DEG-1       RVLIAR.NSF SKLMWGLIIF SFLLMFAYQA SKLIFKFSAH EKITDISLKF DDVEFPAITF CNLNPYKKSL VMMVPSIRDT MDVYDNAKTH SKSBGEKKKP K.........
αrENaC      RLVCSKHNRM KTAFWAVLWL CTFGMMYWQF ALLFEEYLSY PVSLNINLNS DKLVFPAVTV CTLNPYRYT. .....EIKEE LEELDR.... .......... ..........
βrENaC      RI.ICE.GPK KKAMWFLLTL LFACLVCWQW GVFIQTYLSW EVSVSLSMGF KTMNFPAVTV CNSSPFQYS. .....KVKHL LKDLYK.... .......... ..........
γrENaC      RIVVSR.GRL RPLLWIAFTL TAVALIIWQC ALLVFSF..Y TVSVSIKVHF QKLDFPAVTI CNINPYKYS. .....AVSDL LTDLDS.... .......... ..........
Consensus        N     A W L L         Q        Y        I L F D   FPA T  CNLNPYK S           L   D
            221
MEC-4 C.e.  PAPTTKPARR RGKRDLSGAF FEPGFARCLC GSQGSSEQED KDEEKEEELL ETTTKKVFNI NDADEEWDGM EEYD.NEHYE NYDVEATTGM NMMEECQSER TKFDEPTGFD
MEC-4 C.b.  -------S-- ---------- ---------- ---------- --D-----MH ----R-P--- ---------- ----N----- ---------- ---------- ----------
MEC-10      .......... .......... .......... ..DSSSEALE EEEETEYDMN GIT......I QAKRKKRGAG EKGTFEPANS ACECDEEDGS NECEERSTEK .....PSGDN
DEG-1       .......VSR KQHSDASQQM VRELFAK... .......... ...EIEEGMV ELKKSNKTLQ SQNKSGRRRS QRSIENRRYE AIEAHCKCVG NIGMECIRFE SPPRDPSS..
αrENaC      .......... .......... .......... .......... ...ITEQTLF DLYKYNSSYT RQAGARRRSS RD.....LLG AFPHPLQRLR TP........ ..........
βrENaC      .......... .......... .......... .......... ...LMEAVLD KILAPKSSH. ....TNTTST LNFT...IWN HTPLVLIDER NP........ ..........
γrENaC      .......... .......... .......... .......... ...ETKQALL SLYGVKESRK RREAGSMPST LEGTPPRFFK LIPLLVFNEN EK........ ..........
Consensus                                                      E E  L                                             N
            331                 CRDI
MEC-4 C.e.  DRCICAFDRS THDAWPCFLN GTWETTECDT CNEHAPCTKD NKTAKGH... .RSPCICAP. S........R FCVAYNGKTP PIEIWTYLQG G.....TPT. ..........
MEC-4 C.b.  ---------- ---------- ---------- ---------- ---------- ---------- ---------K ---------- ---------- ---------- ----------
MEC-10      DMCICAFDRQ TNDAWPCHRK EQWTNTTCQT CDEHYLCSKK AKKGTKRSEL KKEPCICESK G........L FCIKHEHAAM VLNLWEYFGD SEDFSEIST. ..........
DEG-1       .KCICTYDRD MEVAWPCFNI SVWYDHECPL CHDDGYCEST LPSGTTSSD. .KWPCMCRNR GDTSERDDTP YCIGKAGVGK IEIRKLWLEN NMTTTSTTTT TTTTPPPTTT
αrENaC      .......PPP ....YSGRTA RSGSSSVRD. .NNPQVDRKD WKIGFQLCNQ NKSDCFYQTY S......... .......... .......... .......... ..........
βrENaC      .......DHP VVLNLFGDSH NSSNPAPGST CN...AQGCK ..VAMRLCSA NGTVCTFRNF T......... .......... .......... .......... ..........
γrENaC      .......GKA RDF.FTGRKR KISGKIIHKA SNVMHVHESK KLVGFQLCSN DTSDCATYTF S......... .......... .......... .......... ..........
Consensus          D                         CN                   C
            441                                                                                             CRDII
MEC-4 C.e.  .......... .......... ....EDPNFL EAMGFQGMTD EVAIVTKAKE NIMFAMATLS MQDRERLSTT KRELVHKCSF NGKACDIEAD FLTHIDPAFG SCFTFNHNRT
MEC-4 C.b.  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------V-- ----------
MEC-10      .......... .......... ....EER... EALGFGNMTD EVAIVTKAKE NIIFAMSALS EEQRILMSQA KHNLIHKCSF NGKPCDIDQD FELVADPTFG NCFVFNHDRE
DEG-1       STTTTTTTTP PPTTTARPNQ RAIVSNPETI KAMGFQGMTD GVAMLTRAKE NLMFTMAALS DKQRIALSQS KHEFIEMCSF NGKECDIDED FRLHVDPEFG NCFTFNYDVN
αrENaC      .......... .......... ........SG VDAVREWYRF HYINILSRLS DTSPALEEEA LGNFIFTCRF NQAPCN.QAN YSKFHHPMYG NCYTFNDKNN
βrENaC      .......... .......... ........SA TQAVTEWYIL QATNIFSQVL PQDLVGMGYA PDRIILACLF GTEPCS.HRN FTPIFYPDYG NCYIFNWGMT
γrENaC      .......... .......... ........SG INAIQEWYKL HYMNIMAQVP LEKKINMSYS AEELLVTCFF DGMSCD.ARN FTLFHHPMYG NCYTFNNKEN
Consensus                                       A     K      M  LS        S      I  C F NG   CD     F      P  G NC TFN
            551
MEC-4 C.e.  VN.LTSIRAG PMYGLRMLVY VNASDYMP.T TEATGVRLTI HDKEDFPFPD TFGYSAPTGY VSSFGLRLRK MSRLPAPYGD CVPDGKTSD. .YIYSNYE.. YSVEGCYRSC
MEC-4 C.b.  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MEC-10      IF.KSSVRAG PQYGLRVMLF VNASDYLP.T SEAVGIRLTI HDKDDFPFPD TFGYSAPTGY ISSFGMRMKK MSRLPAPYGD CVEDGATSN. .YIYKGYA.. YSTEGCYRTC
DEG-1       NN.YTSSRAG PMYGIRVLLF VNTSDYMS.T SESSGVRLAI HPPTEYPFPD TFGYSAPVGF ASSFGIKKKV MQRLPAPYGE CVETKKVVDR NYIYAGYD.. YHPEGCHRSC
αrENaC      SNLWMSSMPG VNNGLSLTLR TEQNDFIPLL STVTGARVMV HGQDEPAFMD DGGFNLRPGV ETSISMRKEA LDSLGGNYGD CTENGSDVPV KNL...YPSK YTQQVCIHSC
βrENaC      EKALPSANPG TEFGLKLILD IGQEDYVPFL ASTAGARLML HEQRTYPFIR EEGIYAMAGT ETSIGVLLDK LQGKGEPYSP CTMNGSDVAI QNLYSDYNTT YSIQACLHSC
γrENaC      ATILSTSMGG SEYGLQVILY INEDEYNPFL VSSTGAKVLI HQQNEYPFIE DVGMEIETAM STSIGMHLTE SFKLSEPYSQ CTEDGSDVPV TNI...YNAA YSLQICLYSC
Consensus        S   G   YGL     Y V   DY P      G RL I H     PF D   GY A  G     S G         L  PYG C    G          IY  Y     YS   C  SC
            661                 CRDIII
MEC-4 C.e.  FQQLVLKECR CGDPRFPVPE NARHCDAADP IARKCLDARM NDLGGLHGSF ....RCRCQQ PCRQSIYSVT YSPAKWPSLS LQIQLGSCNG .TAVEC..NK HYKENGAMVE
MEC-4 C.b.  ---------- ---------- G--------- V--R------ ---------- ---------- --G------- ---------- ---------- ---------- ----------
MEC-10      FQELIIDRCG CSDPRFPSIG GVQPCQVFNK NHRECLEKHT HQIGEIHGSF ....KCRCQQ PCNQTIYTTS YSEAIWPSQA LNISLGQCEK .EAEEC..NE EYKENAAMLE
DEG-1       FQNGLIDDCS CGDPRFPVPE GYRHCSAFNA TARTCLEKNI GSVGDFHHIT QKMDKCVCKQ SCEEIIHEVT FSCSKWPSGA ..TDLGDCDG MTESEC..EQ YYRLNAAMIE
αrENaC      FQENMIKKCG CAYIFYPKPK GVEFCDYRKQ SSWGYCYYKL QGAFSLDS.. .LGCFSKCRK PCSVINYKLS AGYSRWPSVK SQDWIFEMLS LQNNYTINNK ..RNGVAKLN
βrENaC      FQDHMIHNCS CGHYLYPLPA GEKYCNNRDF PDWAYCYLSL QMSVVQRE.. ..TCLSMCKE SCNDTQYKMT ISMADWPSEA SEDWILHVLS QERDQSSNIT LSRKGIVKLN
γrENaC      FQTKMVEKCG CAQYSQPLPP AANYCNYQQH PNWMYCYYQL YQAFVREE.. .LGCQSVCKQ SCSFKEWTLT TSLAQWPSEA SEKWLLNVLT WDQSQQINKK LNKTDLAKLL
Consensus   FQ   I  C  C      P P  G  C                                     C Q  C      T  S A WPS      L                     A
            771                 MSDII
MEC-4 C.e.  VFYEQLNFEM LTESEAYGFV NLLADFGGQL GLWCGISFLT CCEFVFLFLE TA..YMSAEH NYSLY..... KKKKAEKAKK IASGSF.... .......... ..........
MEC-4 C.b.  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- V--------- ---------- ----------
MEC-10      VFYEALNFEV LSESEAYGIV KMMADFGGHL GLWSGVSVMT CCEFVCLAFE LI..YMAIAH HINQQ..... RIRRRENAAN EY........ .......... ..........
DEG-1       VFYEQLNYEL LQESEAYGLV NLIADFGGHL GLWLGFSVIT VMEVCVLLVD MISLFFKSRH EEKLLRQSTK RKDVPEDKRQ ITVGSGRKSD AFVSI..... ..........
αrENaC      IFFKELNYKT NSESPSVTMV SLLSNLGSQW SLWFGSSVLS VVEMAEL.IF DLLVITLLML LRRFRS.RYW SPGRGARGAR EVASTPASSF PSRFCPHPTS PPPSLPQQGM
βrENaC      IYFQEFNYRT IEESPANNIV WLLSNLGGQF GFWMGGSVLC LIEFGEI.II DFIWITVIKL VASCKGLRRR RPQRPYTGPP PTVAELVEAH TNCVFQPDTT .SCRPNAEVY
γrENaC      IFYKDLNQRS IMESPANSIE MLLSNFGGQL GLWMSCSVVC VIEIIEVFFI DFFSIIARRQ WHKAKDCWAR RQTPPSTETP SS....RQGQ DNPALDTDDD LPTFTSAMRL
Consensus   VFY  LN       ES A    V  LL  FGGQL GLW G SV      E   L
            881
MEC-4 C.e.  .......... .......... .......... .......... .
MEC-4 C.b.  ---------- ---------- ---------- ---------- -
MEC-10      .......... .......... .......... .......... .
DEG-1       .......... .......... .......... .......... .
αrENaC      TPPLALT... APPPAYATLG PSAPPLDSAA PDCSACALAA L
βrENaC      PDQQTLPIPG TPPPNYDSL. RLQ.PLDTME SDSEVEAI.. .
γrENaC      PPAPGSTVPG TPPPRYNTL. RLDRAFSSQL TDTQLTNEL. .
Consensus
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clude the first three NH_2-terminal exons. The deduced 5′ *mec-4* gene structure is detailed in Fig. 2 *A*.

Predicted *mec-4* exon 1 encodes three in-frame ATGs that could serve as the MEC-4 initiation codon (Fig. 1; amino acid positions 1, 4, and 20). The most 5′ ATG seemed likely to be the initiation codon since it would be the first encountered by ribosomes on the *mec-4* message corresponding to the longest *mec-4* cDNA we isolated, it is flanked by a consensus site for translation initiation in *C. elegans* (Krause, 1995), and it is situated at the beginning of a sequence highly conserved between *C. elegans* and *C. briggsae.* Consistent with the assignment of the codon at base pairs 28–30 as the initation codon, we found that substitution of Leu for Met1 (ZB#39, *mec-4*M1L) renders the *mec-4* allele nonfunctional in vivo, whereas substitutions of Leu for Met4 (ZB#40, *mec-4*M4L) or Met20 (ZB#41,

mec-4M20L) did not disrupt *mec-4* activity (Fig. 1). In addition, a *mec-4* allele that encodes two termination codons after Met1 (ZB#42, *mec-4*Met1XX) failed to complement a *mec-4(lf)* allele. Together these data support our identification of Met1 as the likely first residue of the full-length *mec-4* protein.

Comparison of genomic sequences of *C. elegans* and *C. briggsae mec-4* extend one previously identified exon and add three exons to the published *C. elegans mec-4* gene structure (Driscoll and Chalfie, 1991) (the deduced coding sequences of *C. elegans* and *C. briggsae* MEC-4 are compared in Fig. 2 *B*). A key feature of the 271 amino acids added to the previously deduced MEC-4 protein coding sequence is the presence of a hydrophobic domain (amino acids 110–130) theoretically capable of spanning the cell membrane. Notably lacking is an NH_2-terminal hydrophobic signal sequence, which suggests that, if MEC-4 is a plasma membrane protein, the amino terminus should be situated in the cytoplasm. The structural features of MEC-4 and its predicted membrane topology are summarized in Fig. 3.

Anti-MEC-4 Antibodies Specifically Label the Six Touch Receptor Neurons In Vivo and Recognize Glycosylated and Nonglycosylated MEC-4 In Vitro

To characterize MEC-4 expression and topology, we raised polyclonal antibodies against four peptides within MEC-4 (antigenic determinants are indicated in Fig. 3). AbM4(1-69) was raised against nonconserved residues in the NH_2-terminal domain situated before the first hydrophobic domain. AbM4(527-539) and AbM4(552-561) were raised against peptides situated between predicted membrane-spanning domains MSDI and MSDII. AbM4(746-760) was raised against nonconserved residues in the COOH terminus.

All anti-MEC-4 antisera stained the six touch receptor neurons in whole mount preparations of ZB2, a strain that harbors multiple copies of a construct in which MEC-4 amino acids 1–760 are fused to *E. coli* β-galactosidase (whole mount staining with AbM4(1-69) is shown in Fig. 4, *A* and *B*). Localization of MEC-4 to the touch receptor neurons confirms results of the β-galactosidase staining pattern in this transgenic strain (Mitani et al., 1993) and provides further evidence that the identified NH_2-terminus is included in the full-length MEC-4 protein. Stained cells typically exhibit a "halo" appearance, having a nonimmunoreactive center (Fig. 4 *B*). Juxtaposition of photographs of animals coincubated with anti–MEC-4 antibodies and DAPI (which visualizes nuclei; Fig. 4 *C*) supports that MEC-4 is excluded from the nucleus. Optical sectioning did not offer adequate resolution to localize MEC-4 to the plasma membrane in the high copy number *mec-4* strain ZB2. None of our anti–MEC-4 antisera stain wild-type nontransgenic nematodes that harbor the normal gene dosage of *mec-4*, an observation consistent with previous indications that, in wild-type animals, MEC-4 protein is not present at high levels in the touch receptor neurons (e.g., in situ hybridization is sensitive enough to detect the *mec-7* β-tubulin transcript [Mitani et al., 1993] but does not detect *mec-4* transcripts [Luo, S., and M. Chalfie, unpublished data]).

To begin investigation of the biochemical properties of MEC-4, we transcribed *mec-4* in vitro and synthesized the protein in a rabbit reticulocyte lysate translation system. In vitro translation of a cRNA that encodes the FLAG antigen at the MEC-4 amino terminus yielded a 93-kD protein (apparent molecular mass), slightly larger than the predicted molecular mass of 89 kD (Fig. 5 *A*, lane *3*). Translation of unmodified *mec-4* cRNA generated a product of 89 kD, slightly larger than the predicted molecular

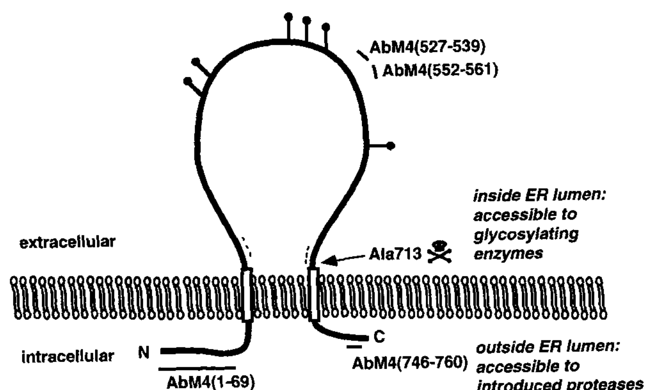

Figure 3. Schematic representation of features of MEC-4 and predicted membrane topology. Membrane-spanning domains are indicated by white boxes, potential sites of N-linked glycosylation are indicated by circles over lines, and the site of dominant death-inducing amino acid substitutions (Ala713) is marked. Regions corresponding to antigenic determinants used to raise anti–MEC-4 antibodies are highlighted by black lines. Dashed lines indicate stretches of hydrophobic residues that might loop back into the membrane as does the H5 domain of voltage-gated K^+ channels. The predicted orientation of domains with respect to the plasma membrane (*left*); predicted environments of MEC-4 domains when *mec-4* is translated in the presence of microsomes and inserted into the ER (*right*).

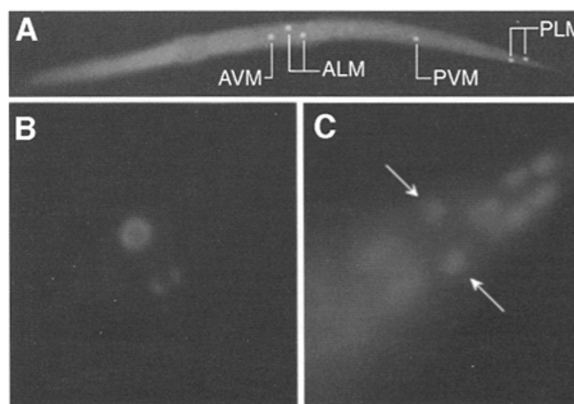

Figure 4. MEC-4 is expressed in the six touch receptor neurons and precluded from the nucleus. (*A*) Immunoreactivity in a whole mount preparation of strain ZB2 incubated with anti–NH_2-terminal MEC-4 antibody AbM4(1-69). ZB2 harbors an integrated array of a *mec-4lacZ* fusion gene (Mitani et al., 1993). Indicated are the six touch receptor neurons. This photograph is a composite of three photographs taken to view different touch cells in the same focal plane. (*B*) Detail of anti–MEC-4 immunoreactivity in the two tail touch receptors, PLML and PLMR. (*C*) DAPI-stained nuclei in the tail of the animal depicted in *B*. (*Arrows*) Touch receptor nuclei. Overlay of photographs in *B* and *C* indicates that the dark inner area typically seen in stained cells corresponds to the nucleus.

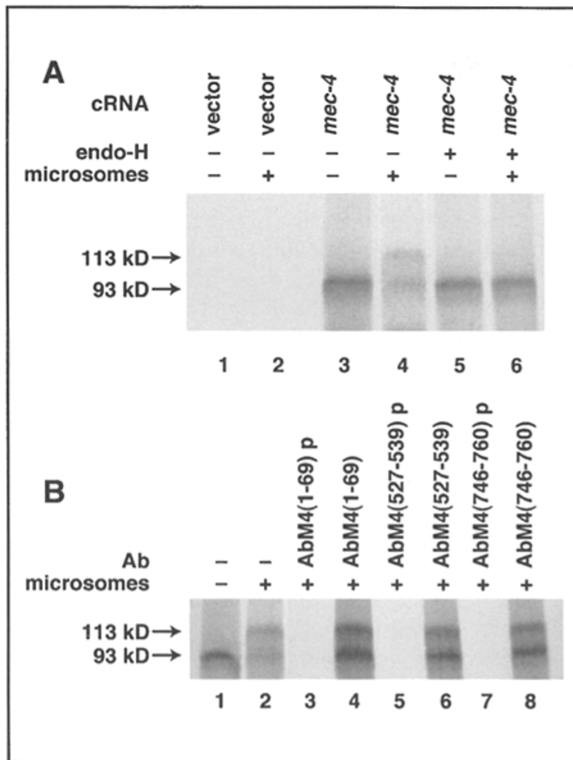

Figure 5. Anti–MEC-4 antibodies immunoprecipitate glycosylated and nonglycosylated forms of MEC-4 synthesized in vitro. (*A*) MEC-4 is glycosylated when translated in the presence of microsomes. cRNA was transcribed from plasmid ZB#46 (*mec-4*) or Bluescript pKS− (*vector*) and translated in the presence or absence of microsomes. Some samples were treated with endo H to remove carbohydrates; all were immunoprecipitated with antibody AbM4(1-69) and resolved using SDS-PAGE. (*B*) Immunoprecipitation profiles of anti–MEC-4 antisera used in this study. *mec-4* cRNA was transcribed and translated as in *A*. Immunoprecipitations were with the antibodies indicated or their preimmune sera (*p*).

mass of 87 kD (data not shown). Protein yields obtained using the *mec-4* cRNA were consistently lower than those obtained using the FLAG–*mec-4* gene. Thus, for biochemical studies presented here, we used the epitope-tagged MEC-4 protein.

Addition of canine pancreatic microsomes to the MEC-4 translation mix resulted in the synthesis of an additional band of 113 kD (Fig. 5 *A*, lane *4*), expected to be a glycosylated derivative of MEC-4. (The first step in N-glycosylation of proteins occurs within the lumen of the microsomal ER and results in the linkage of high mannose oligosaccharide moieties to Asn residues at consensus site Asn-X-Ser/Thr). There are seven consensus sites in MEC-4, five of which are situated between MSDI and MSDII (see Fig. 3). Treatment of the translation products with endo H, which trims off high mannose carbohydrates, restored the product size to 93 kD, confirming that MEC-4 was glycosylated in the microsomal preparation (Fig. 5 *A*, lane *6*). All our anti–MEC-4 antisera immunoprecipitated both nonglycosylated and glycosylated forms of MEC-4 from detergent-solubilized microsomes (Fig. 5 *B*, lanes *3–8*; data not shown for AbM4[552-561]), a result that validates use of these reagents for topology studies. Additional minor bands that were occasionally present in our in vitro translation mix precipitated differentially with various anti–MEC-4 antisera and appear to be MEC-4 degradation products.

Biochemical Probing of the Membrane Topology of MEC-4

To probe MEC-4 membrane topology, we exploited three features of proteins translocated into microsomal membranes: (*a*) only domains situated inside the microsomal lumen can be N-glycosylated at consensus sites; (*b*) domains that project outside the channel lumen are accessible to diffusable protein-modifying reagents such as proteases; and (*c*) topology adopted in the microsomal membrane reflects that of proteins when they reach their final destination in the plasma membrane (Hartmann et al., 1989; Andrews, 1989). To establish which MEC-4 domains are intracellular and which are extracellular, we assayed various domains of the protein for susceptibility to protease digestion when incorporated into microsomal membranes. The predicted MEC-4 topology in the microsomal membrane is noted in Fig. 3.

When we expressed *mec-4* in a transcription/translation system in the presence of microsomes and subsequently treated with proteinase K, a protease-resistant fragment of ~93 kD was produced (Fig. 6 *A*, lane *3*). Protection of this fragment from protease digestion was dependent on the integrity of microsomal membranes, since when microsomes were solubilized with Triton X-100 before proteinase K treatment, the entire product was degraded (Fig. 6 *A*, lane *4*). The protease-resistant fragment could be immunoprecipitated by antibody AbM4(527-539), which recognizes a region predicted to be extracellular (Fig. 6 *A*, lane *6*), but not by anti-NH_2-terminal antibody AbM4(1-69) or anti–COOH-terminal AbM4(746-760) (Fig. 6 *A*, lanes *5* and *7*). The difference in size between the glycosylated MEC-4 protein and the protease-resistant fragment was ~20 kD, a value consistent with proteolytic removal of the predicted 15-kD NH_2 terminus and the 3.4-kD COOH terminus. These findings support the prediction that the NH_2- and COOH-terminal domains normally project into the cytoplasm of the touch receptor neuron.

To confirm that the 93-kD protease-resistant fragment was glycosylated and to get an accurate estimate of its size, we treated the protease-resistant fragment with endo H to remove the carbohydrate residues. This protocol yielded a protein of ~75 kD (Fig. 6 *B*, lane *3*). The segment of MEC-4 that includes the two membrane-spanning domains and the large extracellular domain has a predicted molecular mass of 71 kD. Given that the MEC-4–translated products consistently exhibited slightly higher mobilities than predicted in our in vitro assay, molecular mass values are consistent with the predicted orientation. We conclude that overall MEC-4 topology is as depicted in Fig. 2.

Substitution of a large side-chain amino acid for MEC-4 Ala713 causes swelling and degeneration of the touch receptor neurons (Driscoll and Chalfie, 1991; Ala 442 in the partial protein sequence reported therein). Cognate substitutions for the conserved Ala residue in other *C. elegans*

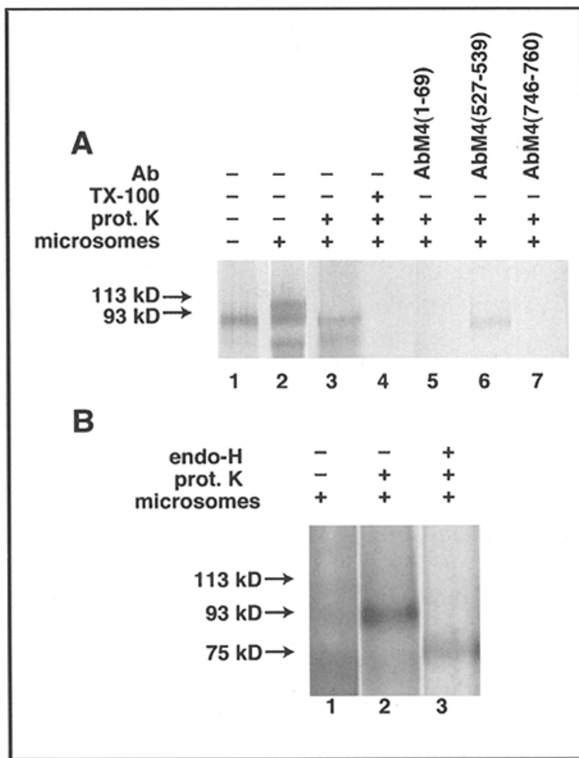

Figure 6. Biochemical assay of MEC-4 topology in microsomal preparations. (*A*) The amino- and carboxy-terminal MEC-4 domains are protease sensitive when the protein is inserted into microsomal membranes. *mec-4* cRNA was transcribed from plasmid ZB#46. *Ab*, anti–MEC-4 antibody used; *TX-100*, solubilized in the presence of Triton X-100; *prot. K*, digested with proteinase K. Lower molecular weight bands in lanes *2* and *3* are MEC-4 degradation products. (*B*) A 75-kD MEC-4 fragment is protected from protease digestion in microsomal preparations. In vitro translation of *mec-4* was as in *A*. Abbreviations are as in legend for *A*; *endo-H*, treated with endoglycosidase H.

family members also induce cell death (Huang and Chalfie, 1994; Shreffler et al., 1995; García-Añoveros et al., 1995), and thus proteins encoded by this family of genes have been named degenerins. We have speculated that such substitutions cause a conformational change that causes the channel to be "locked" open, a condition that disrupts osmotic balance, triggering cell swelling and ultimate death. An A713V substitution in MEC-4 changed neither the protease sensitivity of the NH_2- and COOH-terminal domains nor the size of the proteinase K–resistant domain of MEC-4 (data not shown), suggesting that the overall topology of the protein is unaltered in the toxic form.

An In Vivo Test of Membrane Topology

A complementary strategy that we have taken toward establishing the positioning of MEC-4 domains inside or outside of the touch receptor neurons is based on the observation that β-galactosidase is enzymatically active when situated in the cytoplasm, but when directed out of the cell as a component of a protein fusion, it can become lodged in the membrane, thereby losing activity (Beckwith and Ferro-Novick, 1986; Fire et al., 1990). We constructed a series of gene fusions in which increasing lengths of *mec-4* coding sequence were fused in frame to *lacZ* and assayed their activity in vivo (Fig. 7). To confirm that the β-galactosidase protein was produced in the touch receptor neurons in transgenic animals, we performed whole mount antibody staining using a monoclonal anti–β-galactosidase antibody. Transformants harboring constructs in which *lacZ* was fused to the predicted intracellular domains of MEC-4 exhibited blue-stained touch receptor neurons. In contrast, transgenic lines harboring fusions to the predicted extracellular MEC-4 domain were unable to cleave the X-gal substrate. Specifically, these assays indicate that AA102 and AA760 are inside the cell and AA214 and AA370 are outside the cell, indicating that the MEC-4 topology in vivo corresponds to that deduced in vitro.

Discussion

Three types of stimuli are known to induce opening or closing of ion channels: voltage changes (for voltage-gated channels), binding of chemical transmitters (for ligand-gated channels), and pressure or stretch (for mechanically-gated channels). Understanding the molecular mechanisms of action of mechanically gated channels has lagged significantly behind that of voltage-gated and transmitter-gated channels because genes encoding components of mechanically gated channels have proven difficult to isolate. Here we report the primary sequence and topology of MEC-4, a member of a recently identified family of ion channels that is hypothesized to function as a subunit of a mechanosensitive channel in *C. elegans*.

Comparison of mec-4 Gene and Protein Structure in C. elegans and C. briggsae

We cloned and sequenced the *C. briggsae mec-4* gene to facilitate identification of amino-terminal exons encoding *C. elegans* MEC-4. *mec-4* gene structure is highly con-

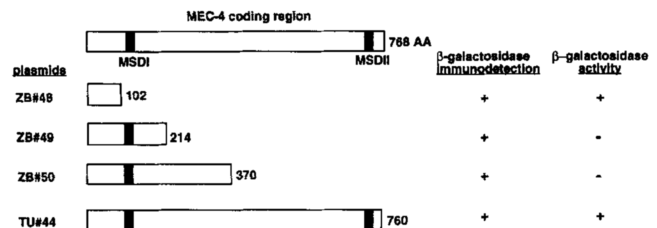

Figure 7. Use of β-galactosidase fusions to test MEC-4 topology in vivo. The full-length MEC-4 coding region, with predicted membrane-spanning domains indicated as black bars, is depicted at top. The number of MEC-4 amino acids encoded in β-galactosidase fusions are indicated at the right. Production of β-galactosidase protein was verified by staining animals with an anti-β-galactosidase antibody (Boehringer Mannheim Biochemicals). Because the antibody staining protocol involves considerable sample loss, we scored staining in populations derived from transgenic parents that harbored extrachromosomal arrays of tested constructs. As a consequence, the population stained included transgenic rollers as well as animals that had lost the transforming DNA. +, at least 10% of animals in the mixed population stained with anti–β-galactosidase antibody. β-galactosidase activity was assayed by permeabilizing selected roller transgenic animals to the chromogenic substrate X-gal. +, β-galactosidase activity in at least 68% of roller transformants; a minimum of 100 animals were scored; −, β-galactosidase activity was not detected in any of a minimum of 134 selected roller animals.

served between *C. elegans* and *C. briggsae*. There are five conserved nucleotide stretches in the 5′ region preceding the likely *mec-4* initiation codon (CRI-V; see Fig. 2 *A*). These may include important sites for gene regulation, although CRI and part of CRII are not required for *mec-4* expression. Interestingly, CRIV includes consensus sites for binding transcription factors UNC-86 and MEC-3, two homeodomain proteins that direct transcription of *mec-4* and other touch cell genes (Way and Chalfie, 1988; Finney et al., 1988; Xue et al., 1992, 1993; M. Chalfie, unpublished observations).

All *mec-4* introns occur at identical positions in the two species, except that intron 12 of *C. elegans mec-4* is absent from the *C. briggsae* gene. The predicted *mec-4* proteins of *C. elegans* and *C. briggsae* are 97% identical (see Fig. 2 *B*), differing by 23 of 768 amino acids (769 in *C. briggsae*). It is striking that more than half of the encoded substitutions are clustered at the 3′ end of exon 3 (*C. elegans* MEC-4 AA192-263), which encodes a region that is highly variable among members of this channel family.

The mec-4 Protein and Relationship to Other Superfamily Members

C. elegans mec-4 encodes a protein of 768 amino acids. A key feature of the 271 amino acids that extend the previously deduced protein coding sequence (Driscoll and Chalfie, 1991) is a hydrophobic domain that spans the cell membrane (MSDI, amino acids 110–130). Genetic and electrophysiological evidence suggest that the more COOH-terminal transmembrane domain, MSDII, plays an important role in the formation of the channel pore (Hong and Driscoll, 1994; Waldmann et al., 1995). Unlike the amphipathic MSDII α helix, the MSDI-predicted α helix is made up of nearly all hydrophobic residues. We suggest MSDI may act as a packing helix that associates with the membrane on one side and with the hydrophobic face of MSDII on the other, such that, in the multimeric channel complex, an internal ring of pore-lining MSDII α helices might be surrounded by an outer ring of MSDI α helices.

The MEC-4 amino terminus could perform a critical role in channel function, perhaps by making cytoskeletal contacts needed for mechanical gating (see further discussion below). The 109 amino acids in the MEC-4 NH_2-terminus include consensus sites for phosphorylation by casein kinase II (AA 39, 56), protein kinase C (AA 34, 39), and tyrosine kinase (AA 36, 41); no other peptide motifs in the Prosite database are present. A short amino acid stretch immediately preceding MSDI (AA 87-95) is highly conserved among nematode and vertebrate family members, suggesting this region plays an important role in channel function.

Our data establish that MEC-4 primary structure is similar to other identified superfamily members from mammals and *C. elegans* (alignment of nematode and rat proteins is shown in Fig. 2 *B*). Family members encode subunits of approximately the same size (638–779 amino acids) that have two predicted transmembrane domains. The Cys-rich domains corresponding to MEC-4 CRDII and CRDIII and short regions preceding and following the two hydrophobic domains are also common to all known superfamily members. The *C. elegans* family members have two distinguishing features: unique to characterized nematode degenerins are an additional Cys-rich domain (CRDI) and a 22–amino acid stretch that has been implicated in channel closing for both the MEC-4 and the DEG-1 degenerins (García-Añoveros et al., 1995).

MEC-4 Transmembrane Topology

Our results confirm models proposed for degenerin topology (Chalfie and Wolinsky, 1990; Driscoll and Chalfie, 1991; Huang and Chalfie, 1994; García-Añoveros et al., 1995) in which both the NH_2 and COOH termini are situated on the cytoplasmic face of the plasma membrane, and a large central domain of the protein, including the Cys-rich domains, the 22–amino acid candidate regulatory domain, and several sites for N-linked glycosylation, projects into the extracellular space. The MEC-4 topology reported here agrees with that determined for the α subunit of the rat amiloride-sensitive Na^+ channel (Renard et al., 1994; Snyder et al., 1994; Canessa et al., 1994*b*) verifying that, in addition to primary sequence homology, *C. elegans* family members share topological similarities with the mammalian channel subunits.

Hydrophobic stretches that project into the membrane from the extracellular side but do not extend out to the cytoplasmic surface would not have been identified by our experiments. Such loops can play critical roles in channel function. For example, in voltage-gated potassium channels, a short hydrophobic H5 segment loops into the membrane and participates in formation of the channel pore (Yellen et al., 1991; Yool and Schwartz, 1991; Hartmann et al., 1991). In the MEC-4 primary sequence, short hydrophobic stretches that are relatively highly conserved are situated after MSDI (AA 148-162) and immediately before predicted MSDII (AA 707-717). Several authors have suggested that an H5-like loop precedes MSDII in members of the epithelial Na^+ channel family (Jan and Jan, 1994; Renard et al., 1994) and in degenerins (García-Añoveros et al., 1995). More refined biochemical analysis of the *C. elegans* degenerins must be coupled with site-directed mutagenesis and electrophysiological studies to establish the localization and functional significance of the short hydrophobic segments.

The finding that large side-chain amino acid substitutions for a conserved Ala residue (MEC-4 AA 713) cause touch cell swelling and death, whereas small side-chain amino acids do not, underlies the hypothesis that bulky side chains at position 713 alter the conformation of the channel, resulting in toxic increased ion influx (Driscoll and Chalfie, 1991). During our investigation of the transmembrane topology of wild-type MEC-4, we also tested the topology of the MEC-4 (A713V). Although we did not find any detectable difference in transmembrane topology for the toxic form of the protein in our in vitro experiments, conformational changes restricted to the membrane or the extracellular side, as well as conformational changes that might occur only in the presence of other channel subunits (such as MEC-10), would not have been detected.

MEC-4 and Models of Mechanosensation

Our working model is that the mechanosensory ion chan-

nel in the touch receptor neurons includes homologous subunits MEC-4 and MEC-10 (Huang and Chalfie, 1994). Genetic data suggest that more than one MEC-4 and more than one MEC-10 subunit are included in the multimeric channel complex (Hong and Driscoll, 1994; Huang and Chalfie, 1994). In addition, since three homologous subunits of the rat epithelial amiloride-sensitive Na^+ are required to reconstitute pharmacological properties of the channel in *Xenopus* oocytes (Canessa et al., 1994*a*), it has been suggested that a third subunit of the touch receptor channel could be encoded by *mec-6*, a gene normally required for touch sensitivity that is also required for *mec-4(d)*- or *mec-10(d)*-induced neurodegeneration (Chalfie and Sulston, 1981; Chalfie and Wolinsky, 1990; Huang and Chalfie, 1994; Canessa et al., 1994*a*).

The topologies of mechanosensitive channel subunits are likely to be of particular significance for channel function. Studies of mechanotransducing channels maintain that tension must be exerted on the channel for regulated opening and closing. For example, the auditory channels of the hair cells of the vertebrate inner ear are thought to act by mechanical gating (for review see Hudspeth, 1989; Pickles and Corey, 1992). These channels appear to be situated toward the ends of specialized stereocilia near attachment sites of the tip-links, thin filaments that connect adjacent stereocilia. When stereocilia are deflected, tension exerted via the tip-links appears to physically pull the channels into an open conformation. For mechanotransduction in *C. elegans*, Chalfie and colleagues have suggested a model in which the touch receptor channels are tethered intracellularly to the specialized touch cell microtubule cytoskeleton by the *mec-2* protein and extracellularly to components of the extracellular matrix (possibly a complex of the *mec-5* and *mec-9* proteins) (Chalfie and Thomson, 1982; García-Añoveros et al., 1995; Huang et al., 1995, Du et al., 1995). Our work establishes that the two Cys-rich domains and the 22–amino acid region within MEC-4 postulated to regulate channel opening (García-Añoveros et al., 1995) are extracellular and thus could be sites of interaction between the channel and the extracellular matrix. Intracellular domains that could associate with MEC-2 or cytoskeletal proteins have also been defined. Thus, now that the topology of MEC-4 (and by extension, the other degenerins) has been determined, specific interactions between the channel and other touch cell components needed for mechanotransduction can be tested.

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