pates directly in lattice contacts, and these crystals dissolve when transferred to low Ca^{2+} solutions.
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13. Nomenclature as in (*6*): AB loops and DE loops separate the antiparallel A and B helices and D and E helices, respectively; numerals identify the domain.
14. The proposed location of the exposed tryptophan at the polar-apolar interface of the phospholipid bilayer is consistent with tryptophan distribution in other membrane proteins. The partitioning of these side chains at the bilayer surface may promote proper ionic interactions and in gramicidin appears to play a role in ion conductance [W. Hu, K.-C. Lei, T. A. Cross, *Biochemistry* **32**, 7035 (1993)].
15. Identical residues in domain 3 are numbered two higher in the human annexin V sequence on account of additional residues.
16. The ϕ,φ angles of Lys^{184} and Trp^{185} as observed in human and rat annexin V structures (rat annexin V numbering) are as follows. Lys^{184}: $\phi = 27°$, $\varphi = 60°$ (human), $\phi = -97°$, $\varphi = 130°$ (rat); Trp^{185}: $\phi = 31°$, $\varphi = 58°$ (human), $\phi = -77°$, $\varphi = 118°$ (rat).
17. The ϕ,φ angles for residues 222 to 224 in human and rat annexin V structures (rat annexin V numbering) are as follows. Residue 222: $\phi = -85°$, $\varphi = 8°$ (human), $\phi = -58°$, $\varphi = -43°$ (rat); residue 223, $\phi = -131°$, $\varphi = 147°$ (human), $\phi = -61°$, $\varphi = -41°$ (rat); residue 224, $\phi = -117°$, $\varphi = 135°$ (human), $\phi = -68°$, $\varphi = -40°$ (rat).
18. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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26. R factor $= \Sigma|F_{obs}(h) - F_{calc}(h)|/F_{obs}(h)$, where $F_{obs}(h)$ and $F_{calc}(h)$ are the observed structure factor amplitude, and the structure factor amplitude calculated from the model, respectively.
27. We thank E. and M. Westbrook for assistance with data collection at Argonne National Labs and G. Petsko for advice and for his crystallographic programs. We thank our colleagues in our laboratories, P. Meers for discussion, and K. Allen for help with figures. Supported by National Institutes of Health grants R29-GM-44554 (B.A.S.), RO1-NS-20357 (J.F.H.), and RO1-DK-41740 (J.R.D.); the American Heart Association, Massachusetts Division, Research Grant-in-Aid 13-539-878 (B.A.S.); and an American Cancer Society Junior Faculty Research Award (B.A.S.). Coordinates have been deposited in the Brookhaven Protein Databank (accession number 1RAN).

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Cooperative Interactions Between the *Caenorhabditis elegans* Homeoproteins UNC-86 and MEC-3

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The POU-type homeodomain protein UNC-86 and the LIM-type homeodomain protein MEC-3, which specify neuronal cell fate in the nematode *Caenorhabditis elegans*, bind cooperatively as a heterodimer to the *mec-3* promoter. Heterodimer formation increases DNA binding stability and, therefore, increases DNA binding specificity. The in vivo significance of this heterodimer formation in neuronal differentiation is suggested by (i) a loss-of-function *mec-3* mutation whose product in vitro binds DNA well but forms heterodimers with UNC-86 poorly and (ii) a *mec-3* mutation with wild-type function whose product binds DNA poorly but forms heterodimers well.

The differentiation of six touch receptor neurons in the nematode *C. elegans* requires two homeobox genes, *unc-86* and *mec-3*. The *unc-86* gene encodes a POU-type homeoprotein required in touch cell precursors to generate the touch receptor neurons (*1*, *2*). The *mec-3* gene, which encodes a LIM-type homeoprotein, is needed to specify the touch cell fate once the cells have been produced (*3*–*5*). The presence of *unc-86* protein (UNC-86) in the touch cells (*2*), the binding of UNC-86 to the *mec-3* promoter, and the requirement for some of the UNC-86 binding sites in vivo for *mec-3* expression (6) suggest that *unc-86* directly initiates *mec-3* expression. Furthermore, mutation of one UNC-86 binding site in the *mec-3* promoter reveals that *unc-86* is also required to maintain *mec-3* expression (6), a role consistent with the finding that UNC-86 is present in the touch cells throughout the life of the animals (*2*).

The *mec-3* gene is required for proper expression of genes such as *mec-4* and *mec-7* (*3*, *7*, *8*) that are needed for touch receptor function (*4*, *9*), and it is required for the maintenance of its own expression (*5*, *6*, *10*). Because *mec-3* protein (MEC-3) binds to its own promoter and mutations in some MEC-3 binding sites affect the maintained expression of *mec-3lacZ* fusions, such autoregulation is likely to be direct (6).

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The proteins UNC-86 and MEC-3 bind to overlapping regions in the *mec-3* promoter (Fig. 1A), two of which (CS2 and CS3) are needed for *mec-3* expression (6). We examined the binding of UNC-86 and MEC-3 to oligonucleotides of these regions in gel mobility–shift assays (*11*). UNC-86 bound differently to the two oligonucleotides, yielding one retarded band with CS2 and two retarded bands with CS3 (Fig. 1B). The two CS3 bands represent the binding of UNC-86 as a monomer and a dimer (*12*). Homodimer formation has been observed with three other POU-type homeoproteins, Pit-1, Oct-2, and Cf1-a (*13*, *14*).

As for MEC-3, it bound to both oligonucleotides and resulted in a single retarded species (Fig. 1B), although the binding of MEC-3 to CS2 was weak. When both MEC-3 and UNC-86 were added to each of the oligonucleotides, the MEC-3 band was reduced and a more intense band (the UM complex) appeared at a position near the UNC-86 monomer band. The UM complex contains both MEC-3 and UNC-86 because it can be supershifted by either an antibody to MEC-3 (anti–MEC-3) or an antibody to an influenza virus epitope tag added to UNC-86. In the UM complex, MEC-3 bound at least 24 times as much oligonucleotide (CS2 or CS3) as it did without UNC-86. The binding of oligonucleotide by UNC-86 in the UM complex increased at least sixfold over the binding of DNA to UNC-86 alone. These data suggest that the binding of both proteins to DNA, especially the binding of MEC-3 to DNA, is greatly increased by a cooperative protein interaction. Although the UM complex is observed in gel shifts with both oligonucleotides, most of the following experiments were done with the CS3 oligonucleotide.

Interactions between UNC-86 and MEC-3 were also detected by chemical cross-linking (*15*) (Fig. 2A), which showed that a heterodimer is formed, and by immunoprecipitation (*16*) (Fig. 2B), which showed that the proteins can interact without DNA.

Truncated UNC-86 and MEC-3 allow for visualization of the heterodimers in gel shifts and help define the domains needed for these interactions (Fig. 3). The POU domain of UNC-86 [UNC-86(POU)] is sufficient for both DNA binding and heterodimer formation. For MEC-3, a region of only 76 amino acids (amino acids 217 to 293) containing the homeodomain and the adjacent COOH-terminal 16 amino acids was sufficient for DNA binding and heterodimer formation, whereas the MEC-3 homeodomain (amino acids 217 to 277) alone bound DNA but formed heterodimers with UNC-86 on DNA very poorly. The LIM domains, which neither bind DNA nor form heterodimers, appear to inhibit DNA binding. The extrahomeodomain re-

gion of MEC-3 required for heterodimer formation differs in two respects from the recently identified region at the amino end of the homeodomain of the yeast α2 protein that has a similar function (*17*): it differs in sequence from the region of α2 and it does not convey heterodimer-forming ability to an *engrailed* homeodomain (*18*). Analysis of deletion mutations and hybrid homeoproteins in *Drosophila* has suggested that the target specificity of these proteins resides in the homeodomain and regions close to it (*19*). Perhaps these extrahomeodomain regions are also needed for protein interactions.

The increase of DNA binding affinity when both UNC-86 and MEC-3 are present could result from an increase in the association of these proteins with DNA or a decrease in their dissociation from it. Because the association of these proteins with DNA is rapid (even at low temperature), we have been unable to measure differences in association rates (*18*). Competition experiments, however, suggest that a significant proportion of the change in affinity can be explained by a decrease in the dissociation rate of the heterodimer from DNA. When a 200-fold excess of unlabeled CS3 oligonucleotide was added to equilibrated complexes with MEC-3 and UNC-86(POU) (to distinguish the different complexes), the MEC-3:DNA and [UNC-86(POU)]$_2$:DNA complexes dissociated immediately (Fig. 4A). The UNC-86(POU) and the MEC-3:UNC-86(POU):DNA complexes, however, were much more stable: some complexes remained 15 min after the addition of unlabeled oligonucleotide. Similar results were observed when full-length UNC-86 was used (*18*).

When the COOH-terminal acidic domain was bound by anti–MEC-3 (Fig. 4B) or was deleted (*20*), we observed an even larger increase in the binding stability of the heterodimer. This result suggests that the binding stability of UNC-86 to DNA is also increased by heterodimer formation.

We used several mutants to examine the in vivo importance of DNA binding and heterodimer formation for *mec-3* function. The *u99* mutation produces a loss-of-function phenotype (9) and causes a cysteine to be replaced by a tyrosine within the 16–amino acid extrahomeodomain region that is required for heterodimer formation (*21*). The predicted protein, MEC-3(C279Y), made in *Escherichia coli*, bound to CS3 as well as wild-type MEC-3, but formed heterodimers with UNC-86(POU) less well (a sixfold reduction) (Fig. 5). This defect is DNA-dependent because MEC-3(C279Y) coprecipitated UNC-86 as well as wild-type MEC-3 (Fig. 3). Thus, the decreased ability of MEC-3(C279Y) to interact with UNC-86 on DNA may underlie the loss of *mec-3* activity produced by this mutation (the touch-insensitive phenotype).

In the mutant protein MEC-3(Q266C), the glutamine at the ninth amino acid in the third helix of the homeodomain was replaced by cysteine. Amino acids at this position have been shown in various ho-

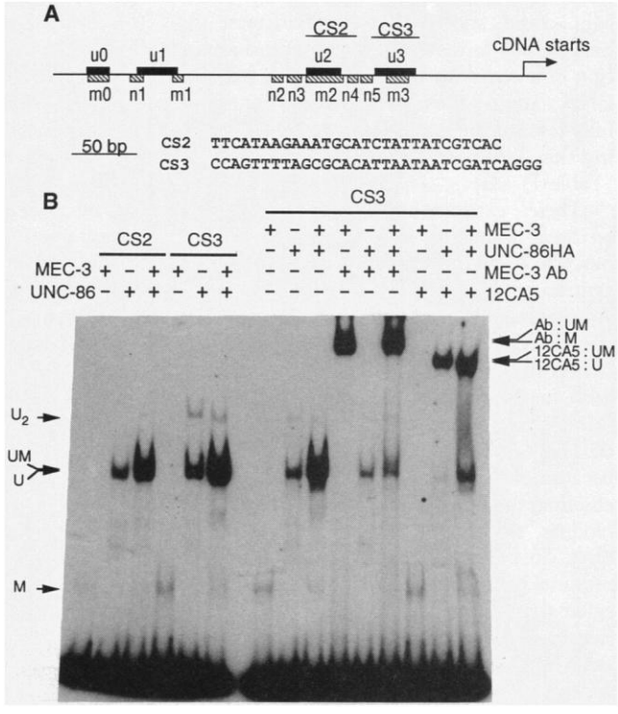

Fig. 1. Mobility-shift assays with MEC-3 and UNC-86. (**A**) Diagram of MEC-3 and UNC-86 binding sites on the *mec-3* promoter. The *mec-3* promoter and its cDNA starts are indicated with a central line and an arrow. The sequences and positions of conserved sequences CS2 and CS3 are shown. Hatched boxes indicate regions protected by MEC-3 in the DNase I footprint, whereas filled boxes indicate regions protected by UNC-86. Data are from (*6*). (**B**) Both UNC-86 and MEC-3 form complexes on CS2 and CS3. UNC-86 binds to CS2 as a monomer but to CS3 as a monomer and a homodimer. UNC-86 forms heterodimeric complexes with MEC-3 on CS2 and CS3. We incubated 1 ng of UNC-86, 0.5 ng of UNC-86HA, and 50 ng of MEC-3 with labeled CS2 or CS3 probes. The epitope-tagged UNC-86 (UNC-86HA) behaved similarly to UNC-86 in gel shifts. U, UNC-86 or UNC-86HA; M, MEC-3; Ab, MEC-3 antibody; 12CA5, anti-influenza virus hemagglutinin, thus, UNC-86HA antibody; U_2, homodimer; and UM, heterodimer.

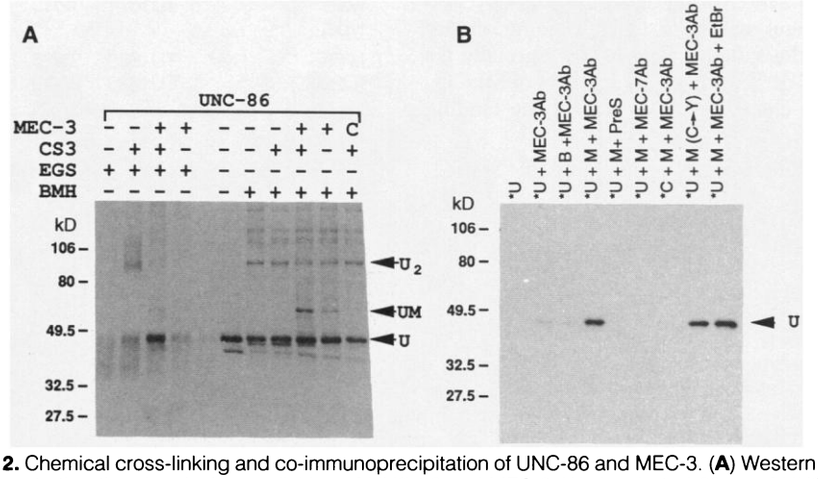

Fig. 2. Chemical cross-linking and co-immunoprecipitation of UNC-86 and MEC-3. (**A**) Western blot demonstrating the chemical cross-linking of UNC-86 and MEC-3. We incubated 60 ng of UNC-86 either alone or with 100 ng of MEC-3(214–321) or with 100 ng of bacterial protein control (C) in the absence (−) or presence (+) of CS3 (5 ng). Bands of UNC-86 monomer (U), homodimer (U_2), and UNC-86:MEC-3(214–321) heterodimer (UM) were detected with an antibody to UNC-86 (*2*). *Bis*-maleimidohexane (BMH) cross-linked UNC-86:MEC-3(214–321) heterodimers much better than ethylene glycol*bis*(succinimidylsuccinate) (EGS) did, but it also produced a higher background of nonspecific bands. (**B**) Co-immunoprecipitation of UNC-86 and MEC-3. UNC-86 (*U) or a control lysate (*C, translated with control mRNA) was labeled in vitro with ^{35}S-methionine and incubated either alone, with 150 ng of bacterial control protein (B), with 150 ng of MEC-3 (M) purified from bacteria extracts, or with 150 ng of MEC-3(C279Y) [M(C→Y)]. MEC-3 antibody (MEC-3Ab), MEC-7 antibody (MEC-7Ab), or preimmune serum (PreS) was added later. In the last lane, ethidium bromide (EtBr) (200 ng/μl) was included.

meoproteins to be critical for the specificity of DNA binding (*22*). The DNA binding of MEC-3(Q266C) was reduced about 10-fold, yet this mutant protein formed heterodimers more readily with UNC-86(POU) than MEC-3 did, showing a twofold increase in heterodimer formation (Fig. 5). Transformation of a *mec-3* mutant strain with genomic DNA carrying the *mec-3*(Q266C) mutation fully rescued the mutant phenotype, indicating that MEC-3(Q266C) functions in vivo (Table 1) (*23*).

These experiments suggest that heterodimer formation is important for *mec-3* function in vivo. Although the cellular concentration of MEC-3 is unknown, our results suggest that cells can better tolerate a reduction in the ability of MEC-3 to bind DNA than a reduction in the ability of MEC-3 to form heterodimers. Heterodimer formation, however, requires the binding of both UNC-86 and MEC-3 to DNA, at least at CS2, because either a mutation in this site that eliminates MEC-3 binding but not UNC-86 binding, or a mutation that greatly reduces UNC-86 binding but not MEC-3 binding, prevents heterodimer formation (Fig. 6). Because the maintenance of *mec-3lacZ* expression in vivo is abolished by these CS2 mutations (6), the binding of both proteins at CS2 appears to be needed, perhaps because heterodimer formation is required, for the maintenance of *mec-3* expression. Because we cannot independently mutate UNC-86 and MEC-3 binding at CS3 (6), we do not know whether the binding of both proteins is required at this site.

The interaction of MEC-3 and UNC-86 increases both the DNA binding affinity and the stability of binding, especially for MEC-3. With limiting amounts of MEC-3, these changes should increase the binding specificity of MEC-3 by sequestering it to regions that bind UNC-86. Such sequestration appears to have occurred in our deoxyribonuclease I (DNase I) footprinting of the *mec-3* promoter with MEC-3 and UNC-86 (6). At least at CS2 (where we can separate UNC-86 and MEC-3 binding), the interactions of MEC-3 with DNA and with UNC-86 are required for heterodimer formation. Thus, specificity could be increased by the presence of a larger binding surface provided by both UNC-86 and DNA.

The increased specificity resulting from the interaction with UNC-86 may be particularly important for the function of MEC-3 in vivo. Like other POU-type homeoproteins (*24*) and in contrast with MEC-3 and most other homeoproteins (*25*), UNC-86 shows considerable DNA binding specificity (6). This binding specificity and the expression of UNC-86 during development before MEC-3 (*2*, *5*) may allow UNC-86 to act like a coupling protein, recruiting MEC-3 to regions where it has already bound. Because many homeodomain proteins have similar DNA binding properties in vitro, the suggestion has been made that other factors are needed to increase DNA binding specificity so that these widespread proteins can specify cell fate (*25*). Our results provide direct support for this hypothesis.

Protein-protein interactions involving homeoproteins have been reported but, except for the homeodomain-like proteins a1 and α2 of yeast (*26*), not between two very different homeoproteins like UNC-86 and MEC-3. Interactions between POU-type homeoproteins (*10*, *27*, *28*) and between homeoproteins and nonhomeoproteins (*29*–*31*) have

Table 1. Transformation of *mec-3* DNAs. The indicated *mec-3* DNAs and *dpy-20*(+) DNA were microinjected into *mec-3(e1338) dpy-20(e1282)*; these nematodes are dumpy (Dpy) and touch-insensitive (Mec) at 25°C. Normal-length germ line transformants were then scored for touch sensitivity. Only one of the TU1671 animals was touch insensitive at both the head and tail.

DNA	Amino acid at position 266	Strain	Fraction touch sensitive	
			Head	Tail
pTU23 (wild type)	Gln	TU1670	15/16	15/16
		TU1671	12/19	18/19
TU#55 (Q266C)	Cys	TU1689	25/25	25/25
		TU1690	28/29	28/29

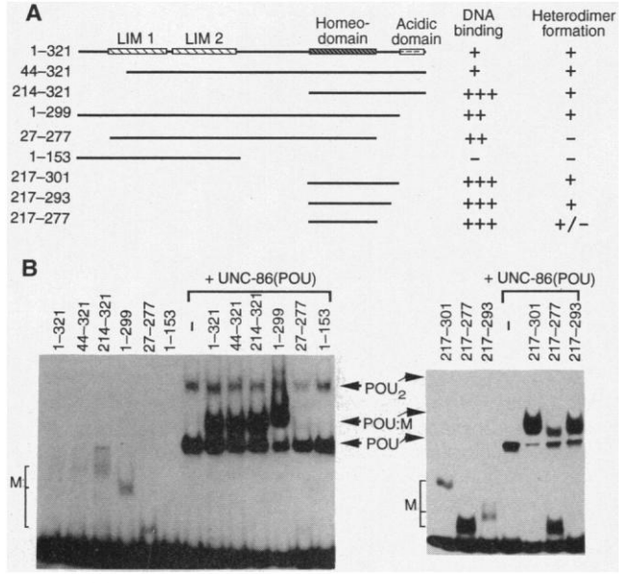

Fig. 3. DNA binding and protein interactions of MEC-3 deletion mutants. (**A**) Diagram of the *mec-3* protein deletions. DNA binding was scored as wild type (+), absent (−), or increased (++ or +++). Heterodimer formation was scored as wild type (+), absent (−), or greatly reduced (+/−). (**B**) Mobility-shift assays of UNC-86(POU) with different MEC-3 deletions. Incubations included 0.5 ng of UNC-86(POU) and 100 ng of MEC-3 proteins [except that 5 ng of MEC-3(214–312) was used]. The MEC-3(217–301), MEC-3(217–293), and MEC-3(217–277) peptides used in these experiments (at 5 ng) were made by cleavage of purified GST-fusion proteins (*34*).

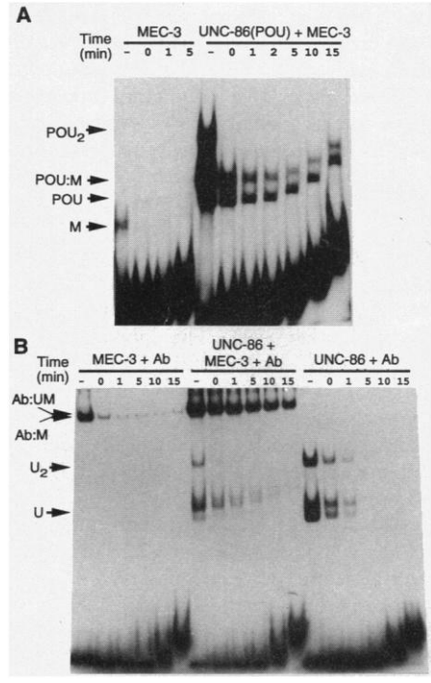

Fig. 4. Dissociation of MEC-3:DNA, UNC-86:DNA, and MEC-3:UNC-86:DNA complexes. (**A**) Heterodimer formation stabilizes MEC-3 binding to DNA. A 200-fold excess of unlabeled CS3 was added to 350 ng of MEC-3 alone or to 350 ng of MEC-3 and 20 ng of UNC-86(POU) that had been preincubated with labeled CS3. Aliquots were loaded at the indicated times (min) onto a continuously running 5% nondenaturing polyacrylamide gel. (**B**) An antibody that binds the acidic tail of MEC-3 also increases the DNA binding stability of the UNC-86:MEC-3 heterodimer but not of MEC-3. Because the complex MEC-3:Ab bound more DNA than MEC-3 alone (Fig. 1B), the antibody appears to increase MEC-3 DNA binding affinity but not its binding stability. A 400-fold excess of unlabeled CS3 was added to 350 ng of MEC-3, 14 ng UNC-86, or 350 ng of MEC-3 and 14 ng UNC-86 that had been preincubated with MEC-3 antibodies and labeled CS3.

been described, and some of these interactions alter DNA binding specificity (*26*, *30*).

In vivo, UNC-86 and MEC-3 are likely to be only part of a larger DNA binding complex. The LIM repeats and the acidic COOH-terminus of MEC-3, which do not appear to be required for DNA binding or heterodimer formation, may bind to other factors. LIM repeats, which in MEC-3 appear to inhibit DNA binding, permit protein-protein interactions (*32*), and acidic domains often promote transcriptional activation by interacting with cellular proteins (*33*). One consequence of the latter interaction suggested by our stability studies is that the masking of the negative charge of the MEC-3 COOH-terminus could further stabilize the binding of the protein complex.

Our results extend the model for touch cell differentiation derived from the analysis of touch mutants (*9*). Instead of being required solely for the expression of *mec-3* in these cells, *unc-86* also appears to be needed to maintain this expression. Because UNC-86 is found immunologically throughout the life of the cells (*2*) and *unc-86* is required for the expression of *mec-7* (*7*), UNC-86, perhaps as part of a heterodimer with MEC-3, could be involved in more downstream transcriptional events.

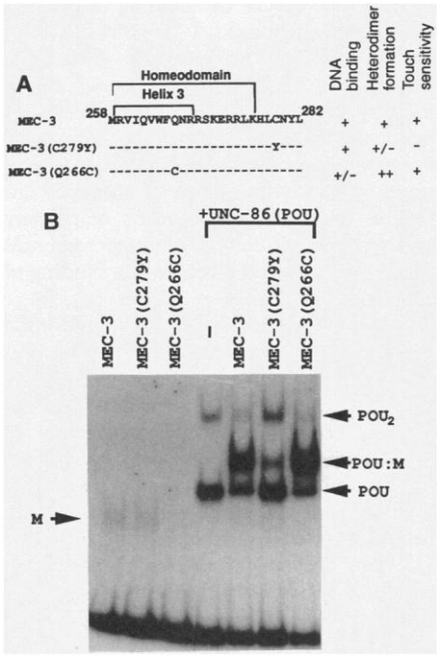

Fig. 5. Effects of *mec-3* mutations. (**A**) Diagram of mutations in *mec-3* and their effects on DNA binding, heterodimer formation on DNA, and in vivo phenotype. DNA binding and heterodimer formation were either at wild-type (+), reduced (+/−), or increased (++) amounts. For the in vivo phenotype, the nematodes were either touch-sensitive (+) or -insensitive (−). (**B**) Mobility-shift assays with wild-type and mutant MEC-3. We used 100 ng of MEC-3 and 0.5 ng of UNC-86(POU).

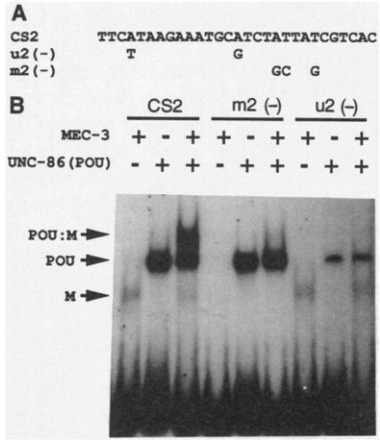

Fig. 6. Binding of MEC-3 and UNC-86 to CS2, m2(−), and u2(−). (**A**) Diagram of the mutations in CS2. Only the changed nucleotides are indicated. (**B**) Mobility-shift assays using 300 ng of MEC-3 and 0.5 ng of UNC-86(POU).

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11. Gel mobility shift assays were carried out as described [M. G. Peterson, N. Tanese, B. F. Pugh, R. Tjian, *Science* **248**, 1625 (1990)] with oligonucleotides (Operon) except that 0.5% NP-40, 4 μg of bovine serum albumin (BSA), and 1 μg of poly(dG-dC) were used in the incubation buffer, and EDTA and $MgCl_2$ were omitted. The gel-shifted bands were quantitated with a Molecular Dynamics phosphoimager. We used two antibodies for the antibody supershifting experiments: an affinity-purified rabbit antibody to MEC-3 (J. C. Way, S. Mitani, M. Chalfie, unpublished data), which recognizes the acidic domain of MEC-3, and the monoclonal antibody 12CA5 (Harvard Monoclonal Facility), which recognizes the nine–amino acid hemagglutinin tag of the influenza virus [J. Field *et al.*, *Mol. Cell. Biol.* **8**, 2159 (1988)]. The tag is attached to the NH_2-terminus of UNC-86 to make UNC-86HA. These antibodies were added 30 min before the addition of radioactive probes in the antibody supershifting experiments. Proteins were made in *E. coli* with standard molecular biology procedures [F. S. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989)]. A Sac I–Hind III cDNA fragment, which contains the whole open reading frame (ORF) of the *mec-3* gene (*6*), was subcloned into pBluescript KS(+). Further in vitro mutagenesis [T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985)] added an Nde I site at the *mec-3* initiator methionine codon and a Bam HI site immediately after the stop codon to produce plasmid TU#54. In vitro mutagenesis of TU#54 resulted in DNA encoding the MEC-3 deletions, which were inserted into pET-3a [A. H. Rosenberg *et al.*, *Gene* **56**, 125 (1987)]. The UNC-86(POU) expression vector contains amino acids 114 to 303 of UNC-86, which was amplified by the polymerase chain reaction (PCR) [R. K. Saiki *et al.*, *Science* **239**, 487 (1988)] from pET-3a–UNC-86 (*2*) into pET-3a. The influenza HA tag was added by PCR to make the UNC-86HA expression vector. Expression of various *unc-86* and *mec-3* proteins was as described (*6*), except that expression was induced in the cell line BL21 (DE3) Lys S [F. W. Studier and B. A. Moffat, *J. Mol. Biol.* **189**, 113 (1986)]. The various *unc-86* proteins were purified from inclusion bodies as described by D. A. Hager and R. R. Burgess [*Anal. Biochem.* **109**, 76 (1980)]. UNC-86 prepared in this way showed no apparent difference in the gel-shift assay as compared with UNC-86 prepared previously (*6*). We purified *mec-3* proteins from inclusion bodies as described [A. Kumagai and W. G. Dunphy, *Cell* **64**, 903 (1991)], with the following modifications: 1 mM EDTA and 200 mM dithiothreitol (DTT) were added to the initial denaturing buffer, and the protein preparations were dialyzed against buffer RB [0.2 M tris-HCl (pH 8.2) and 0.5 M NaCl] containing 0.4 M L-arginine, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione [J. Buchner and R. Rudolph, *Biotechnology* **9**, 157 (1991)] and against 100 mM KCl, 50 mM tris-HCl (pH 7.9), 10% glycerol, and 0.1% NP-40. The MEC-3 proteins, except for MEC-3 (214–321), prepared in this way were detected as a single band by Coomassie blue staining.
12. The putative homodimer band appeared only at higher UNC-86 concentrations. We observed heterodimer bands in gel-shift experiments when we used full-length UNC-86 and a truncated form of UNC-86 [UNC-86(POU)], which only contains the complete POU domain. We also observed these bands in chemical cross-linking experiments (*16*) with full-length UNC-86.
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16. We synthesized ^{35}S-Met–labeled UNC-86 in vitro with a rabbit reticulocyte lysate (Promega) under conditions suggested by the manufacturer and passed the labeled protein through a Spin-30 column (Clontech). The in vitro–labeled protein was incubated with *mec-3* proteins purified from *E. coli* and appropriate antibodies in RIPA buffer [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988)] with 1 mM DTT, 1 mM spermidine, and BSA (0.2 μg/μl). Antibodies were precipitated with protein A–agarose (Boehringer Mannheim). In some experiments ethidium bromide (200 ng/μl) was added to prevent DNA-protein interaction as described [J. S. Lai and W. Herr, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6958 (1992)].
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20. In experiments where MEC-3 lacks the acidic tail [MEC-3(1–299)], the affinity of MEC-3(1–

299):UNC-86 to DNA is about 11 times that of MEC-3(1–299) alone (Fig. 2). In stability experiments the half-life of MEC-3(1–299) binding is less than 20 s, whereas that of MEC-3(1–299):UNC-86 binding is 5 min, a change that could account for most of the change in affinity.
21. Eleven *mec-3* alleles (*4, 8, 9*) were sequenced. The 2.8-kb *mec-3* genomic fragment that contains the whole *mec-3* coding sequence was amplified from single-mutant animals according to B. D. Williams, B. Schrank, C. Huynh, R. Shownkeen, and R. H. Waterston [*Genetics* **131**, 609 (1992)] and inserted into pBluescript KS(−). We pooled and sequenced DNAs from at least four independent transformants. The mutations found were the following [nucleotide position as in (*4*); amino acid position as in (*6*)]: *e1338*, insertion of an A at position 2762 (W69TAG); *e1498*, C3352T (Q149TAA); *e1612* and *u466*, G3664A (splice donor site abolished); *u6*, C3806T (Q262TAG); *u81*, G3921A (splice donor site abolished); *u99*, G3858A (C279Y); *u184*, G3814A (W264TGA); *u467*, T2082C, G2762A, and deletion of an A at position 3951 (V21A, W69TAG); *u468*, T2036C, C2518T, and G2686A (S6P, Q35TAA, and M44V); and *u469*, G2545A (splice donor site abolished).
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34. We generated the glutathione *S*-transferase (GST) fusion proteins GST–MEC-3(217–301), GST–MEC-3(217–293), and GST–MEC-3(217–277) by inserting the appropriate PCR fragments into the pGEX-2T vector (Pharmacia). The resulting proteins made in BL21(DE3)Lys S were purified according to Smith and Johnson (*30*) and cut with thrombin as described in (*31*) to produce the MEC-3 polypeptides.
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Prevention of Collagen-Induced Arthritis with an Antibody to gp39, the Ligand for CD40

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The ligand for the CD40 antigen is a 39-kilodalton protein, gp39, expressed on the surface of activated CD4$^+$ T cells and is essential for thymus-dependent humoral immunity. The role of gp39-CD40 interactions in autoimmune disease was investigated in vivo with the use of an antibody that blocks their interactions (anti-gp39). Arthritis induced in mice by immunization with type II collagen was inhibited by anti-gp39. Anti-gp39 blocked the development of joint inflammation, serum antibody titers to collagen, the infiltration of inflammatory cells into the subsynovial tissue, and the erosion of cartilage and bone. Thus, interference with gp39-CD40 interactions may have therapeutic potential in the treatment of autoimmune disease.

Experimental evidence has established that the CD40 ligand, gp39, is essential in the development of thymus-dependent immunity (*1*). The expression of gp39 is largely restricted to activated CD4$^+$ T cells (*2–4*), and its receptor, CD40, is a mitogenic receptor expressed on mature B cells (*5*). It has been postulated that expression of gp39 during cognate T cell–B cell interactions is an essential signal in the development of thymus-dependent (TD) immunity. This postulate has been proven with the recognition that X-linked hyper immunoglobulin M (IgM) syndrome (HIM), which results in the severe reduction of thymus-dependent responses and Ig isotype switching, results from point mutations in the gp39 gene (*6–9*). Mutant gp39 proteins from HIM patients cannot trigger B cell activation or bind to CD40 (*6*). Although TD humoral immune responses in HIM patients are impaired, cell-mediated immune responses are intact, suggesting that gp39 is only essential in antibody-mediated immunity. A monoclonal antibody (mAb) specific to murine gp39, MR1, blocks the binding of gp39 to CD40 and also blocks T helper (T_H) cell–dependent B cell activation in vitro (*10*). Experiments were designed to test whether a mAb specific to gp39 could be used therapeutically to control the course of humoral immune responses in normal and disease states. The administration of anti-gp39 interferes with the development of primary and secondary humoral immune responses (*11*), apparently by blocking the binding of gp39 to CD40.

The effects of anti-gp39 administration on a murine model of human autoimmune disease, rheumatoid arthritis, are presented herein to determine the role of interactions between gp39 and CD40 in the development of autoimmune disease. Collagen type II–induced arthritis (CIA) is induced in susceptible strains of mice by intradermal injections of heterologous native type II collagen (CII) (*12, 13*). Because the transfer to naïve animals of antibodies to CII leads to a condition histopathologically different from CIA and results in only transient synovitis (*14*), the role of humoral immunity in the development of CIA is not completely understood. In contrast, the transfer of both antibodies to CII and CD4$^+$ T cells from mice immunized with denatured CII completely reconstitutes the symptoms of classical arthritis (*15*). Therefore, synergy between the cellular and humoral arms of the immune response appears to be essential in the development of arthritis.

The kinetics of disease progression that result from the immunization of DBA/1J (*H-2^q*) mice with CII are depicted in Fig. 1. Mice were immunized with chick CII in complete Freund's adjuvant (CFA) and then challenged 3 weeks later with soluble CII. After this challenge, a dramatic rise in the rate of disease progression was observed. To quantitate the course of disease progression, we assigned scores to mice depending on the severity of inflammation of the fore- and hindlimbs. Three groups of mice (eight per group) were immunized with CII in CFA, and 1 week later each group received either no mAb, anti-gp39, or irrelevant hamster Ig (HIg) (Fig. 1). Mice were subsequently administered antibody (250 μg per mouse) every 4 days until the end of the experiment. This antibody treatment regime was used because previous titrations showed that this regime of mAb administration inhibited more than 90% of the primary (IgM) anti–sheep red blood cell response (*11*). Antibody half-life is estimated to be 12 days, so that injections every 4

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