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Synergistic activation of transcription by UNC-86 and MEC-3 in *Caenorhabditis elegans* embryo extracts

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The nematode *Caenorhabditis elegans* has been a choice organism for the study of developmental regulation using classical and molecular genetic approaches. Consequently, many genetically defined pathways have been described and numerous regulatory genes have been identified. However, the biochemical and functional properties of these putative transcription factors have remained uncharacterized, partly because *C.elegans* cell-free transcription reactions have not been developed. Here we describe the *in vitro* transcriptional activation properties of two *C.elegans* homeodomain proteins, UNC-86 and MEC-3, in nuclear extracts derived from *C.elegans* embryos. Whereas the POU homeodomain protein, UNC-86, alone was able to activate transcription of the *mec-3* promoter *in vitro*, the LIM homeodomain protein, MEC-3, failed to bind DNA or activate transcription on its own. However, in the presence of both UNC-86 and MEC-3, we observed cooperative binding to the *mec-3* promoter and synergistic activation of transcription *in vitro*. Protein–protein interaction assays revealed that UNC-86 can bind directly to MEC-3, and *in vitro* transcription studies indicate that both proteins contain a functional activation domain. Thus, formation of a heteromeric complex containing two activation domains results in a highly potent activator. These studies provide direct functional evidence for coordinated transcriptional activation by two *C.elegans* DNA binding proteins that have been defined genetically as regulators of gene expression during embryogenesis.

Keywords: *Caenorhabditis elegans*/MEC-3/synergism/transcriptional activation/UNC-86

Introduction

It is well established that regulation of transcription plays a major role during development of eukaryotic organisms. Progress in our understanding of transcriptional control during development has resulted, in large measure, from molecular genetic studies of model organisms including *Drosophila melanogaster*, *Caenorhabditis elegans* and mice. Although many advances have come from elegant genetic dissection of regulatory pathways governing development in these model organisms, it is also apparent from studies in *Drosophila* that *in vitro* biochemical strategies can provide a powerful complement to the genetic studies (reviewed in Lawrence and Morata, 1994; Perrimon and Desplan, 1994). The development of *in vitro* biochemical assays that recapitulate the function of transcription factors directing developmental events is of particular value when the genetic pathways and regulatory gene products have been identified. For example, the DNA binding and transcriptional activation properties of *Drosophila* homeotic and segmentation genes (Desplan *et al.*, 1985; Hoey and Levine, 1988; Thali *et al.*, 1988; Schier and Gehring, 1992), as well as anterior–posterior polarity genes (Ingham, 1991; Nüsslein-Vohlard, 1991), contributed significantly to our understanding of the regulatory mechanisms that control these complex processes. Thus, it has become increasingly apparent that the molecular events underlying gene regulation in eukaryotes are best attacked by a combination of genetics and biochemistry.

Although genetic studies of *C.elegans* have kept pace with other model organisms, the biochemistry and functional analysis of regulatory genes and gene products in the worm have lagged behind. Recent advances in identifying and cloning of regulatory genes, as well as characterization of developmental pathways in *C.elegans*, have provided important reagents for developing functional biochemical assays. It, therefore, seems reasonable to revisit the prospects of devising *in vitro* transcription reactions that would faithfully mimic the promoter recognition properties of the endogenous transcriptional machinery. In an effort to establish *in vitro* transcription, we have used nuclear extracts derived from *C.elegans* embryos. We have chosen the *mec-3* promoter as a model DNA template for these studies, because its primary structure has been determined (Way *et al.*, 1991; Xue *et al.*, 1992) and the developmental and tissue-specific expression of this transcription unit has been documented (Way and Chalfie, 1988, 1989; Chalfie and Au, 1989). Genetic experiments reveal that two putative transcriptional regulators, UNC-86 and the product of the *mec-3* gene itself, are required for proper expression in a set of sensory neurons that respond to a gentle touch stimulus (Way and Chalfie, 1988, 1989; Chalfie and Au, 1989; Finney and Ruvkun, 1990). During early development, UNC-86 is expressed before MEC-3 and is thought to help initiate the expression of the *mec-3* gene (Way and Chalfie, 1989). The presence of both UNC-86 (Finney and Ruvkun, 1990) and MEC-3 (Way and Chalfie, 1989) proteins in the specialized 'touch' cells, as well as the recent analysis of these proteins in binding specific promoter regions of the *mec-3* gene (Xue *et al.*, 1992, 1993), support the hypothesis that they may act as transcriptional regulators. The *unc-86* gene product is a broadly expressed prototype POU homeodomain protein (Finney *et al.*, 1988) that is thought to regulate the transcription of many target

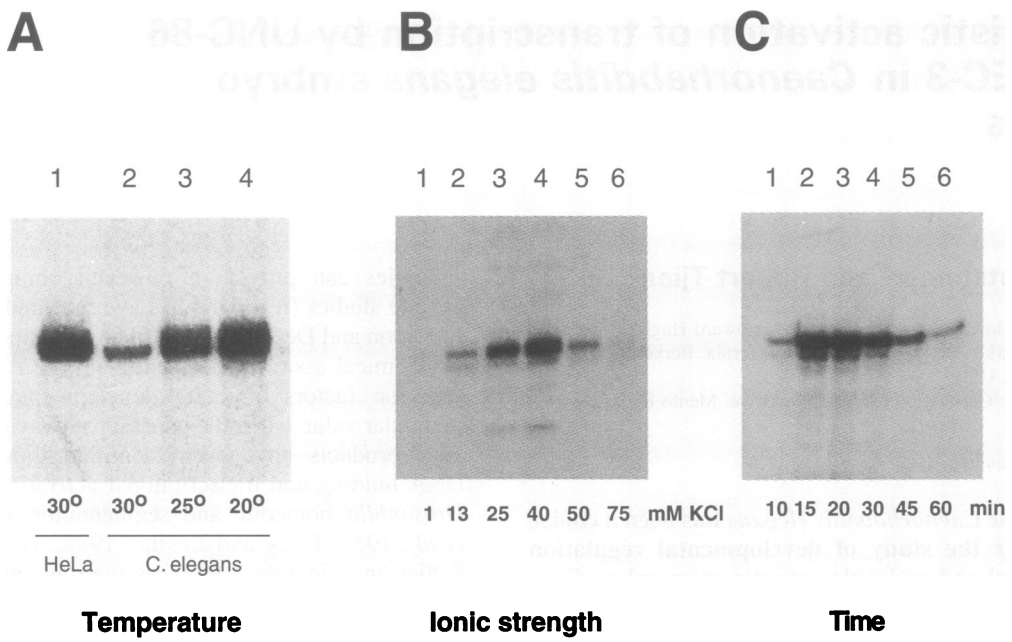

Fig. 1. Optimization of transcription conditions in *C.elegans* nuclear extracts. (**A**) The adenovirus major late promoter was used for *in vitro* transcription in a HeLa nuclear extract at 30°C (lane 1) or in a *C.elegans* nuclear extract either at 30°C (lane 2), 25°C (lane 3) or 20°C (lane 4). (**B**) The optimal KCl concentration for the transcription of the *C.elegans hsp16-2* gene was assayed by primer extension using a *C.elegans* nuclear extract. The transcription reactions were performed for 30 min at 20°C in the presence of the indicated concentration of KCl. (**C**) Accumulation of the transcription products originating from the *hsp16-2* promoter was assayed by primer extension in a *C.elegans* nuclear extract at 25°C and in a final KCl concentration of 40 mM. The disappearance of transcripts starting after 20 min is likely to be due to the presence of RNase activity in the crude nuclear extract.

genes, including *mec-3*. By contrast, MEC-3 is a LIM-homeoprotein (Way and Chalfie, 1988) that directs cellular differentiation and appears to specify neuron formation. Interestingly, the product of the *mec-3* gene appears to autoregulate its own transcription and is expressed in the specialized neuronal 'touch' cells (Way and Chalfie, 1989). Several other genes including *mec*-7 and *mec*-4 are involved in the differentiation of 'touch' cells, and are also likely targets of the MEC-3 and UNC-86 proteins.

In this paper, we report the ability of two genetically defined *C.elegans* regulators, MEC-3 and UNC-86, to activate transcription synergistically *in vitro*. In addition, we provide evidence for specific interactions between these two transcriptional regulators that lead to the formation of a heteromeric complex with enhanced transcriptional activation and DNA binding properties.

Results

A C.elegans in vitro transcription system

The development of transcriptionally active extracts from the nematode *C.elegans* has been hindered by several factors inherent in the anatomy of the organism. *C.elegans* is protected by a tough cuticle and, consequently, the isolation of intact nuclei presents a formidable challenge. In addition, the adult worm gut contains numerous potent proteases that impede preparation of active transcription extracts. Indeed, many past attempts to generate transcriptionally active extracts from adult worms had failed. Our previous experience with *Drosophila* suggested that embryos may provide a better source for the production of active transcription extracts (Heiermann and Pongs, 1985; Biggin and Tjian, 1988; Soeller *et al.*, 1988). To obtain nuclear extracts derived from *C.elegans* embryos, we have undertaken a multi-step procedure, first isolating embryos from gravid hermaphrodites and then isolating nuclei prior to the preparation of transcription extracts. The worms are grown in synchronized liquid cultures and harvested when the adults are bloated with embryos. The embryos are subsequently released from the worms by treatment with bleach (see Materials and methods), washed extensively and collected by centrifugation. Since whole embryonic extracts did not yield material suitable for *in vitro* transcription (data not shown), we purified nuclei from the isolated embryos. Unfortunately, the chitinous shell, which protects *C.elegans* embryos, is extremely resistant to most conventional forms of cell disruption procedures. Having first tried a variety of enzymatic, chemical and mechanical breakage procedures without success, we eventually settled on the use of a metal dounce homogenizer fitted with a tight metal pestle which efficiently disrupts the embryonic shell without substantially damaging the nuclei. After homogenization of the embryos, the nuclei are isolated from cytoplasmic components and soluble nuclear proteins are extracted in a highly concentrated form for *in vitro* transcription reactions (see Materials and methods).

In our initial attempts to test the transcriptional properties of embryo nuclear extracts, we used the well characterized adenovirus major late promoter (AdML), which has been found to direct a high level of core promoter activity in most *in vitro* transcription systems. Under conventional HeLa transcription conditions of 30°C, G-less analysis of *in vitro* synthesized AdML RNA revealed a rather weak signal with the *C.elegans* nuclear extract when compared with the HeLa nuclear extract (Figure 1A, lanes 1 and 2).

However, when the reaction temperature was lowered to mimic the optimal growth temperatures of *C.elegans* (i.e. 20–25°C), the transcriptional activity of the *C.elegans* extract increased dramatically (Figure 1A, lanes 3 and 4). Indeed, transcription under these lowered temperature conditions was comparable with that of HeLa extracts. Next, we tested the ability of the *C.elegans* embryo extract to direct transcription from an endogenous worm promoter. For these experiments, we used the heat shock *hsp 16-2* gene promoter. Maximal basal transcription was optimized by varying the ionic strength and the time course of reactions. We found that a 20 min incubation period at 25°C in the presence of 40 mM KCl produced optimal levels of basal transcription. These conditions were adopted for all subsequent experiments described in this report.

Although *in vitro* transcripts measured by G-less cassette analysis suggested a unique and presumably accurate start site utilization under our *in vitro* transcription conditions, we wanted to confirm that the actual start site of transcription initiation observed *in vitro* corresponded to the 5′ ends of *in vivo* transcripts. We, therefore, compared the *in vitro* transcription start sites of two *C.elegans* genes (*hsp 16-2* and *her-1*) directly with their *in vivo* start sites by primer extension analysis and found that they were coincident (data not shown). Thus, the embryo extract is apparently capable of initiating transcription efficiently and accurately from both TATA-containing (*hsp 16-2*) and TATA-less (*her-1*) promoters.

Having established that the embryo extracts are not only transcriptionally active but also able to discriminate between core promoter sequences and random DNA, we proceeded to assess the ability of the transcription system to respond to sequence-specific activators. First, we chose to test well characterized mammalian transcription factors, such as Sp1, VP16 and c-Jun, each one representing a different class of activation domains (i.e. Gln, acidic, etc.). In the case of VP16 and c-Jun, we used chimeric proteins consisting of the DNA binding domain of the yeast protein, Gal4, fused to the activation domain of VP16 (Gal4–VP16) or c-Jun (Gal4–JunA2). The addition of either Gal4–VP16, Gal4–JunA2 or Sp1 to the *C.elegans* nuclear extract enhanced the level of transcription initiation substantially (Figure 2). The most dramatic activation (40-fold) was observed with Gal4–VP16, while Gal4–JunA2 and Sp1 directed a somewhat more modest transcriptional enhancement (~10-fold). These levels of transcriptional activation are comparable with the *in vitro* enhancement typically induced by promoter-specific activators in HeLa and *Drosophila* extracts. Thus, it appears that at least three different classes of activators are able to mediate transcriptional activation in the *C.elegans* nuclear extracts.

The transcriptional activation properties of UNC-86 in vitro

Given the established involvement of UNC-86 and MEC-3 in the differentiation of neurons and the finding that a delimited promoter of the *mec-3* gene is necessary and sufficient for temporal and cell type-specific regulation, we have chosen this transcription unit for further biochemical analysis using our embryo nuclear extracts. The minimal *mec-3* promoter has been delineated (Way *et al.*, 1991; Xue *et al.*, 1992), and reveals the presence of at least five

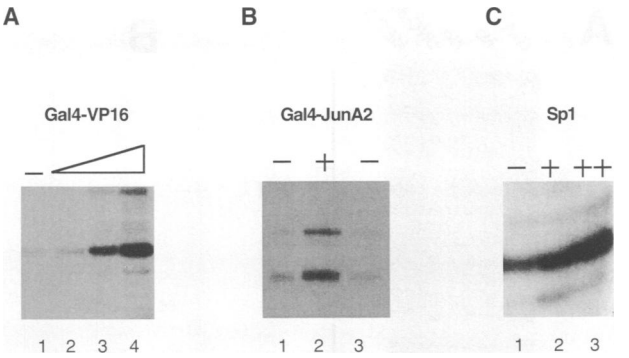

Fig. 2. Activation of transcription by mammalian transcription factors in a *C.elegans* nuclear extract. (**A**) Lane 1, transcription in the absence of Gal4–VP16; lanes 2–4, increasing amount of recombinant Gal4–VP16. The reporter plasmid, G_5E4T, contains five Gal4 binding sites upstream of the E4 TATA box and initiator (Lin *et al.*, 1988). The gel was exposed to a phosphorimager screen for quantification. (**B**) Lanes 1 and 3, no addition of Gal4 fusion protein; lane 2, transcription in the presence of recombinant Gal4–JunA2 protein. (**C**) Lane 1, no addition of Sp1; lanes 2 and 3, addition of increasing amount of purified native Sp1. The reporter plasmid, G_6TI, contains six Sp1 binding sites as well as the TATA box and initiator sequences from the adenovirus major late promoter (Pugh and Tjian, 1990).

potential transcription factor binding sites (CS1–5). A minimal *mec-3* promoter fragment containing ~300 bp of DNA is sufficient to direct the appropriate tissue-specific patterns of *mec-3* expression. Deletion of CS1 and CS2 results in the complete loss of *mec-3* expression (Xue *et al.*, 1992), while a fusion of CS1, 2 and 3 to a heterologous promoter element is sufficient to direct proper cell type-specific expression in transformed worms (Way *et al.*, 1991). We have, therefore, used the minimal *mec-3* promoter containing CS1–3 for our DNA binding and transcription studies. As an initial step in studying the transcriptional regulation of the *mec-3* promoter, we have purified recombinant UNC-86 protein from *Escherichia coli* and tested its ability to bind template DNA sequence specifically and to activate transcription *in vitro*. DNase footprint protection experiments reveal that, in accordance with previously published data (Xue *et al.*, 1992), purified UNC-86 protein recognizes and binds efficiently to the *mec-3* promoter at three sites (Figure 3A). This same preparation of purified UNC-86 protein (Figure 3B) was tested for transcriptional activation in the *C.elegans* nuclear extracts. Using a *mec-3*-derived template containing CS1, 2 and 3 linked to the *hsp 16-2* core promoter region, we observed substantial (10-fold) stimulation of basal transcription by the addition of UNC-86 protein (Figure 3C and D). These results indicate that UNC-86 can bind to and activate transcription of the *mec-3* promoter *in vitro* and suggest that this enhancer binding protein participates in the control of the *mec-3* gene during *C.elegans* development.

MEC-3 and UNC-86 protein interact and bind cooperatively to the mec-3 promoter

It has been reported that MEC-3 and UNC-86 proteins can form heterodimers and bind to DNA (Xue *et al.*, 1993). Having demonstrated the ability of purified UNC-86 to bind to and activate transcription of the *mec-3* promoter *in vitro*, we next set out to test the ability of MEC-3 protein to bind DNA and activate transcription

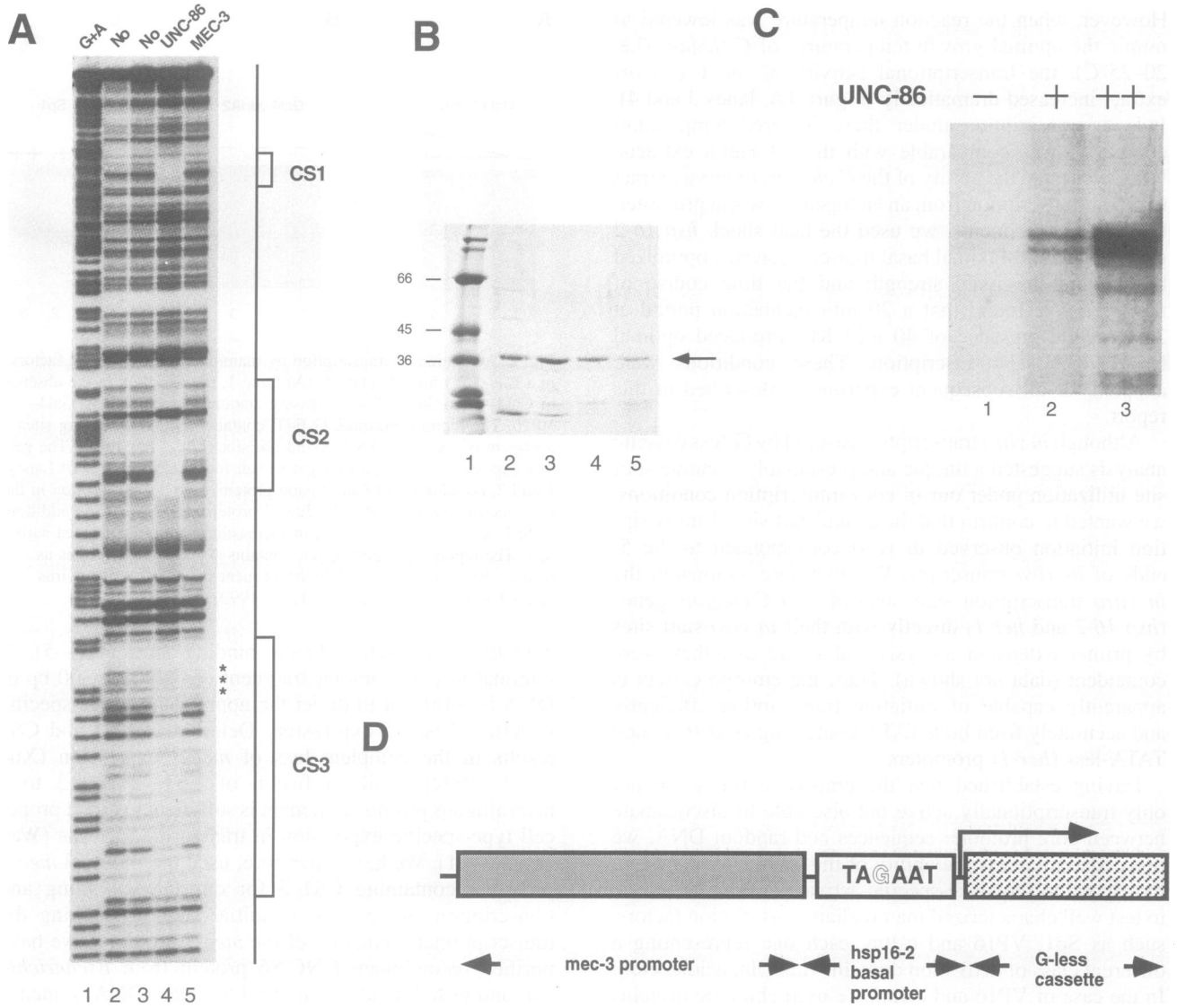

Fig. 3. *In vitro* activation by UNC-86 of the *mec-3* gene in a *C.elegans* nuclear extract. (**A**) Footprinting of the *mec-3* promoter: lane 1, G+A sequencing reaction; lanes 2 and 3, DNase I degradation pattern of the footprinting probe in the absence of recombinant proteins; lane 4, footprinting reaction performed in the presence of UNC-86; lane 5, footprinting reaction performed in the presence of MEC-3. CS = consensus sequence as described by Xue *et al.* (1992). The footprint probe contained the *mec-3* promoter sequence from pSL3007 (see Materials and methods). The plasmid was digested with *Hin*dIII, radiolabeled with Klenow and cut with *Eco*RI. Note that the protection observed in lane 5 and indicted by *** is caused by the presence of the FLAG peptide (DYKDDDK) which was used to elute recombinant MEC-3 from the antibody column. Footprinting reactions performed with the FLAG peptide only gave a similar result to that shown in lane 5 (data not shown). (**B**) Protein gel showing purified recombinant UNC-86 from *E.coli*. Lane 1, molecular weight marker (size in kDa), lanes 2–5, eluted fractions from a DNA affinity column. (**C**) *In vitro* transcription activation by UNC-86. Lane 1, basal level of transcription of the reporter plasmid in the absence of UNC-86; lanes 2 and 3, addition of increasing amount of purified recombinant UNC-86 to a *C.elegans* nuclear extract. (**D**) Representation of the reporter plasmid (pSL 3044) used to assay transcription activation by UNC-86 and MEC-3. The main components are sites CS1–3 from the *mec-3* promoter and the basal promoter from the *hsp16-2* gene fused to a G-less cassette (see Materials and methods).

alone or in combination with UNC-86. In contrast to the results we observed with purified recombinant UNC-86, purified MEC-3 protein isolated from baculovirus-infected Sf9 cells (Figure 4B) failed to bind selectively to the *mec-3* promoter region as determined by DNase I footprint analysis (Figure 4A). This result suggested that MEC-3 may bind directly to UNC-86 to form a heteromeric complex that functions as a potent transcriptional activator. To test this possibility, we have first determined the ability of MEC-3 and UNC-86 to interact with each other. To detect potential protein–protein interactions between MEC-3 and UNC-86, we prepared a column containing purified recombinant MEC-3 covalently linked to agarose beads. A crude bacterial lysate containing recombinant UNC-86 was applied to the MEC-3 affinity column, extensively washed and eluted with 1 M NaCl (Figure 4A). The predominant protein that bound selectively to the MEC-3 resin was UNC-86 and what appears to be proteolytic breakdown products of UNC-86, as determined by silver staining of the SDS–PAGE gel (Figure 4A). The identity of UNC-86 bound to the MEC-3 resin was confirmed by the use of an anti-UNC-86 antibody (data not shown). These results indicate that MEC-3 protein is able to bind UNC-86 *in vitro*.

The specificity and stability of the interaction between MEC-3 and UNC-86 prompted us to determine the effect

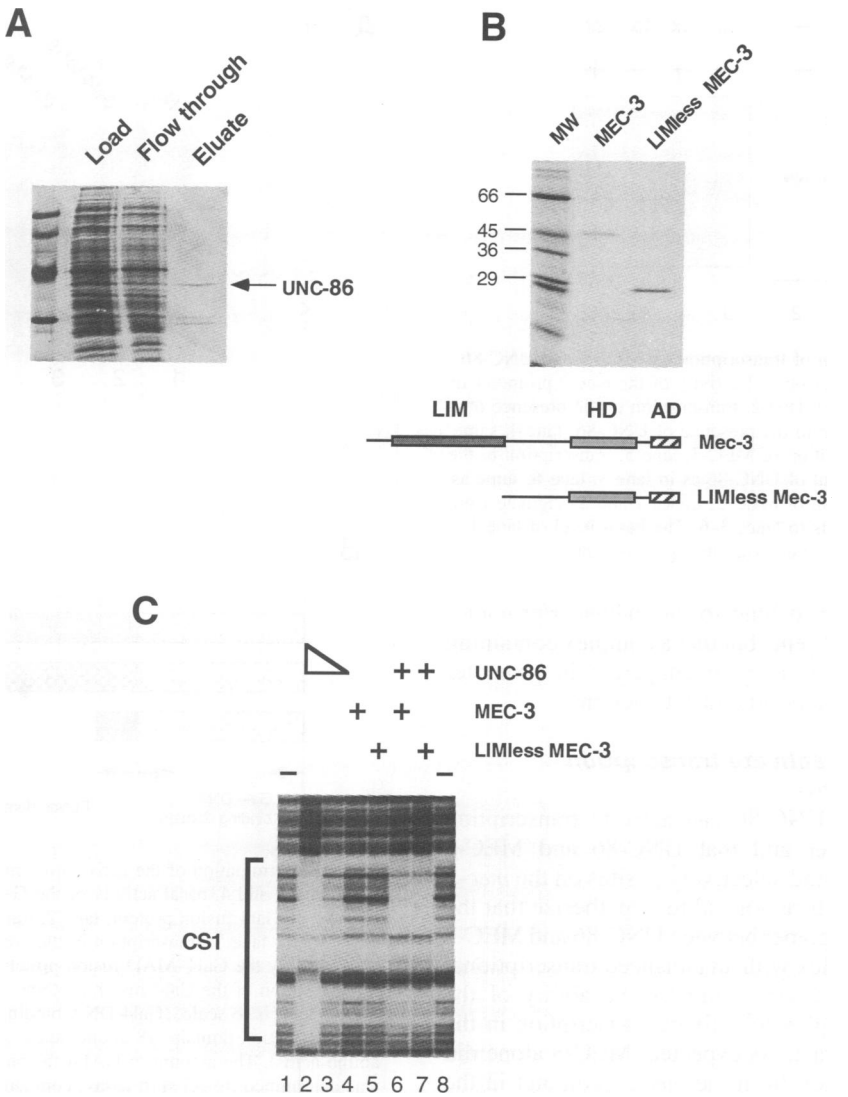

Fig. 4. MEC-3 and UNC-86 interact and bind cooperatively to DNA. (**A**) Silver-stained protein gel of an UNC-86 binding assay. The load is a crude *E.coli* extract containing recombinant UNC-86. In the eluate, the major band corresponds to full-length UNC-86. The two faster migrating bands observed in the lane are degradation products of UNC-86, as determined by Western blotting (data not shown). (**B**) Coomassie-stained protein gel showing immunopurified full-length MEC-3 and LIM-less MEC-3 proteins expressed in Sf9 cells. The MEC-3 primary amino acid sequence can be divided into four regions: LIM domains (aa 27–146), a middle region of unknown function (aa 147–216), the homeodomain (HD) (aa 217–277), and the acidic domain (AD) (aa 300–321). (**C**) Footprinting at the *mec-3* promoter site CS1. Lanes 1 and 8, footprinting reactions in the absence of protein; lane 2, footprinting in the presence of saturating amount of UNC-86 giving complete protection of CS1; lane 3, footprinting reaction in the presence of a low amount of UNC-86; lane 4, footprint in the presence of full-length MEC-3; lane 5, footprint in the presence of LIM-less MEC-3; lane 6, footprint with the same amount of UNC-86 as in lane 3 plus MEC-3 protein: lane 7, footprint with the same amount of UNC-86 as in lane 3 plus LIM-less MEC-3.

of this interaction on the DNA binding properties of UNC-86. For simplicity, we have focused on the CS1 binding site within the *mec-3* promoter for these studies. We have expressed two different versions of the MEC-3 protein, the full-length product and a truncated version lacking the end terminal LIM domain (Figure 4B). Neither full-length MEC-3 nor the LIM-less MEC-3 derivative alone bind to the DNA template (Figure 4C, lanes 4 and 5). As expected, UNC-86 alone can bind to the DNA efficiently (Figure 4C, lanes 2 and 3). However, when either full-length or LIM-less MEC-3 are added to a concentration of UNC-86 that gives a partial or weak footprint we observe an enhanced protected pattern (Figure 4C, compare lane 3 with lanes 6 and 7). Since we showed previously that MEC-3 and UNC-86 interact directly with each other, this apparent enhanced DNA binding is likely to be, at least in part, due to protein–protein interactions between these two subunits. Although UNC-86 can form both homo- and heteroligomers in the presence of MEC-3, we were unable to detect any isologous MEC-3 complexes by co-expressing MEC-3 with different N-terminal tags in Sf9 cells (data not shown). This suggests that MEC-3 is largely a monomer in the absence of UNC-86.

We were surprised to find that the LIM-less MEC-3 protein also failed to bind DNA on its own, since it has been reported that the LIM domains may be responsible for inhibiting DNA binding (Sanchez-Garcia *et al.*, 1993). However, our data indicate that this truncated version of MEC-3 is able to cooperate with UNC-86 in binding to DNA. These results, taken together, suggest that MEC-3

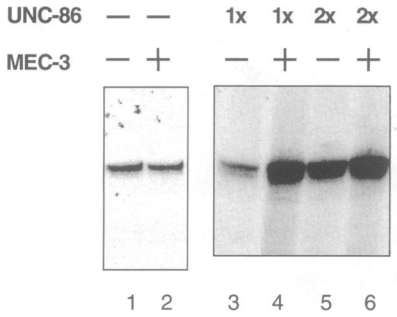

Fig. 5. Synergistic activation of transcription by MEC-3 and UNC-86 *in vitro*. Lane 1, basal transcriptional activity of the *mec-3* promoter in the *C.elegans* nuclear extract; lane 2, transcription in the presence of MEC-3; lane 3, transcription in the presence of UNC-86; lane 4, same as lane 3 except for the addition of MEC-3; lane 5, transcription in the presence of twice the amount of UNC-86 as in lane 3; lane 6, same as lane 5 except for the addition of MEC-3. Lanes 1 and 2 originate from a different set of experiments to lanes 3–6. The basal level of lane 1 should therefore not be compared with the signal of lane 3.

alone may not be able to bind to the enhancer/promoter sequences of the *mec-3* gene, but that a complex containing MEC-3 and UNC-86 is able to cooperate in promoter binding via direct protein–protein interactions.

MEC-3 and UNC-86 activate transcription synergistically in vitro

We have shown that UNC-86 can activate transcription of the *mec-3* promoter and that UNC-86 and MEC-3 together cooperate to bind selectively to sites on the *mec-3* promoter. It was, therefore, logical to hypothesize that the formation of a heteroligomer between UNC-86 and MEC-3 might result in a complex with an enhanced transcriptional activity. Therefore, we tested directly the ability of the MEC-3–UNC-86 complex to activate transcription in the *C.elegans* nuclear extract. As expected, MEC-3 alone did not activate transcription from the *mec-3* promoter in the reconstituted transcription system, consistent with the inability of MEC-3 to bind to the template DNA on its own (Figure 5, lane 2). To assay the contribution of MEC-3 to transcriptional activation in the presence of UNC-86, we added MEC-3 to limiting amounts of UNC-86, which alone would ordinarily give a low or moderate level of activation (Figure 5, lane 3). When amounts of MEC-3 protein, which alone were unable to activate transcription, were added to transcription reactions containing sub-optimal concentrations of UNC-86, we observed an 8-fold stimulation of transcription (Figure 5, lane 4). At higher concentrations of UNC-86, we observed a more modest increase in activation (3-fold) on addition of MEC-3 (Figure 5, lane 6). These results suggest that, although MEC-3 alone does not activate transcription, the protein can act synergistically with UNC-86 to stimulate transcription of the *mec-3* promoter *in vitro*.

The acidic domain of MEC-3 can behave as an activation domain

Since MEC-3 is unable to bind DNA or activate transcription on its own but rather binds DNA and activates transcription through a cooperative interaction with UNC-86, we asked whether MEC-3 contained an activation domain. The primary amino acid sequence of MEC-3 reveals a highly acidic C-terminal region that is reminiscent

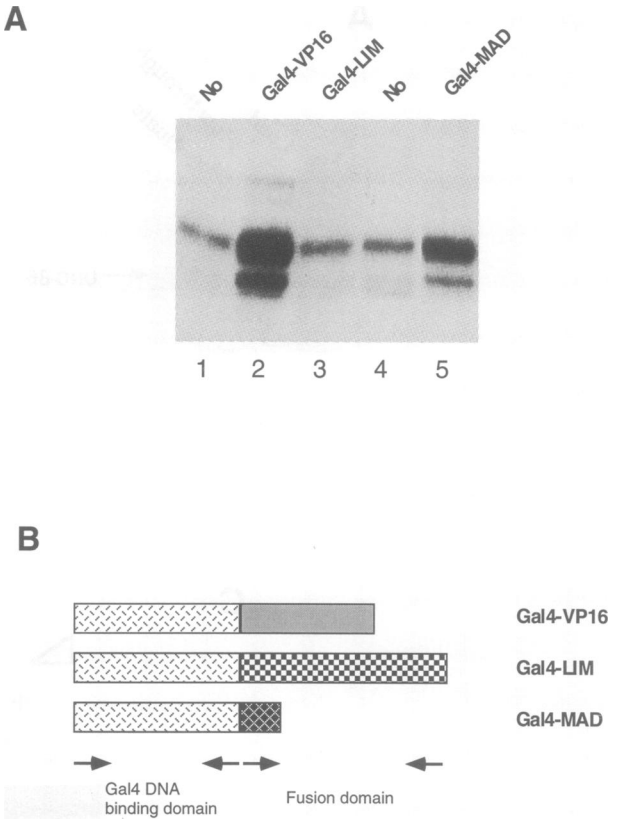

Fig. 6. Determination of the activation domain of MEC-3 *in vitro*. (**A**) Lanes 1 and 4, basal activity of the G_5E4T promoter in the absence of Gal4 fusion protein; lane 2, transcription in the presence of Gal4–VP16; lane 3, transcription in the presence of Gal4–LIM; lane 5, activation by the Gal4–MAD fusion protein. (**B**) Schematic representation of the Gal4 fusion proteins used in Figure 5A. Note that the drawing is to scale. (Gal4 DNA binding domain, 94 amino acids; VP16 activation domain, 78 amino acids; MEC-3 acidic.domain, 22 amino acids). The amount of GAL4 fusion proteins used in the assay was determined by gel shift assay; comparable GAL4 fusion DNA binding activities were used in the transcription assay.

of the 'acidic' class of activation domains found in many transcription factors (Way and Chalfie, 1988). We, therefore, assayed the ability of this C-terminal 22 amino acid domain to serve as a transcriptional activator when fused to the heterologous DNA binding domain of Gal4. This fusion protein Gal4–MAD (MEC-3 acidic domain) was tested for transcriptional activation of a DNA template containing multiple Gal4 binding sites (Figure 6B). The Gal4–MAD protein, when added to the *C.elegans* embryo extract, stimulated transcription significantly (5-fold) above basal levels of activity (Figure 6A, lane 5). The activation by Gal4–MAD was not as strong as that observed with the prototypic Gal4–VP16 activator (Figure 6A, lane 2). However, the level of transcriptional activation by the Gal4–MAD protein was substantially above basal transcription (Figure 6A, lane 1) or a Gal4–LIM domain fusion protein which apparently lacks activation function under our *in vitro* conditions (Figure 6A, lane 3). These results suggest that the C-terminal acidic domain of MEC-3 can serve as an activation domain. Thus, it is possible that when MEC-3 and UNC-86 form a heteroligomer, the MEC-3 protein not only serves to enhance the DNA binding properties of UNC-86, but the acidic activation

domain of MEC-3 may also contribute to the transcriptional enhancement properties of the heteroligomeric complex.

Discussion

Here, we report the development of an *in vitro* transcription system derived from *C.elegans* embryos. Initial characterization of *C.elegans* nuclear extracts indicates that the efficiency of accurate transcription is comparable with typical HeLa or *Drosophila* extracts. As expected, the *C.elegans* extracts are able to direct transcription from both homologous and heterologous promoters. Similarly, *C.elegans* promoters are likely to be at least partially active in HeLa and *Drosophila* extracts. However, it appears that endogenous templates behave in a more consistent and reproducible manner in homologous *in vitro* reactions. This is particularly evident when attempting to study multiple regulatory components in order to decipher complex control mechanisms during embryogenesis and development. Thus, an *in vitro* transcription reaction derived from *C.elegans* embryos should provide a useful biochemical tool for studying transcriptional regulation during early development, and could also serve as a more general transcription system.

As a preliminary test of the fidelity of *C.elegans* embryo extracts, we have characterized the promoter-specific transcriptional activation properties of two regulatory proteins, UNC-86 and MEC-3. Using an endogenous promoter (*mec-3*) as a template, we found that UNC-86 and MEC-3 proteins can act synergistically to activate transcription *in vitro*. As expected, UNC-86 was able to bind promoter sequences selectively and activate transcription in the absence of MEC-3. By contrast, MEC-3 failed to bind DNA and was transcriptionally inactive in the absence of UNC-86. DNA binding studies suggest that UNC-86 and MEC-3 may bind cooperatively to their cognate recognition sequences. Thus, it appears that both the DNA binding capability and transcriptional activation functions of MEC-3 are dependent on a nucleoprotein complex containing UNC-86.

Studies of sequence-specific transcription factors have revealed a number of cases in which formation of heteroligomers is required in order to produce a functional complex. For example, the AP-1 proteins (c-Jun and c-Fos) collaborate to form an active AP-1 complex containing heterodimers of Jun–Fos, whereas Fos alone is unable to form dimers, bind DNA or activate transcription on its own (Turner and Tjian, 1989). Similarly, the yeast transcription factors α2 and **a**1 cooperate to form a complex in which a C-terminal peptide of α2 interacts with **a**1 to induce a complex active for DNA binding and transcriptional activation (Goutte and Johnson, 1993). Thus by analogy to Fos and **a**1, the MEC-3 protein requires a partnership with UNC-86 to form an active DNA binding species capable of transcriptional activation. The mechanism by which UNC-86 and MEC-3 cooperate to form an active complex on DNA is presently unknown. However, it appears that the DNA binding properties of MEC-3 are somehow dependent on an interaction with UNC-86. It is, therefore, tempting to draw a parallel between MEC-3–UNC-86 and **a**1–α2. However, it is also possible that the failure of MEC-3 to bind DNA in the absence of UNC-86 is due to an inability of MEC-3 to form isologous oligomers. If so, then the MEC-3–UNC-86 situation would be more similar to the Fos–Jun case.

It is curious that by using native baculovirus-expressed protein we failed to detect any sequence-specific DNA binding activity of MEC-3 alone, whereas in a previous study (Xue *et al.*, 1993) using denatured and renatured bacterially expressed MEC-3 some DNA binding activity was detected. Initially we hypothesized that the failure of full-length native MEC-3 to bind DNA might be due to the N-terminally located LIM domain which has been postulated to act as a potential negative regulatory domain (Sanchez-Garcia *et al.*, 1993). However, a truncated LIM-less MEC-3 protein also failed to bind DNA. Interestingly, both full-length and LIM-less MEC-3 proteins were active for DNA binding as well as synergistic transcriptional activation in the presence of UNC-86. Thus, it seemed unlikely that the lack of DNA binding activity by MEC-3 could be attributed solely to the LIM domain. Also, our results suggest that the LIM domain, which has been postulated to serve as a protein–protein interface (Freyd *et al.*, 1990; Sadler *et al.*, 1992), is not essential for interactions with UNC-86 or to direct activation of transcription by MEC-3. Instead, our experiments identify a C-terminal region of MEC-3 which, when fused to a heterologous DNA binding domain, is able to serve as an activation region. Thus, it is possible that the synergistic activation of transcription we observe *in vitro* with MEC-3 and UNC-86 is due in part to cooperative DNA binding and in part to the activation function contributed by MEC-3. We speculate that a MEC-3–UNC-86 heteroligomer provides a combination of activation domains that is more potent than either alone. It is interesting to note that UNC-86 is able to turn on the expression of the *mec-3* gene *in vivo*, but maintenance of *mec-3* expression requires the MEC-3 protein (Way and Chalfie, 1989). This suggests that UNC-86 may serve as a weak activator of the *mec-3* gene and that, after expression of *mec-3*, the gene product combines with UNC-86 to form an activator complex that maintains *mec-3* expression in the few cells required to mediate the response of *C.elegans* to touch. This might represent an ingenious mechanism for positive autoregulation of a genetic pathway during development of the worm.

Materials and methods

Growth of worms and preparation of nuclear extracts

Growth in culture of the Bristol N2 wild-type strain of *C.elegans* was as described (Sulston and Hodgkin, 1988). All embryonic nuclear extracts were prepared from nematodes grown in liquid cultures. Liquid cultures were started by seeding ten 15 cm plates of N2 into 100 ml of complete S medium [S medium supplemented with trace metals and 1× PSM (Gibco) and 1× nystatin (Gibco)] in a 250 ml fluted flask supplemented with *E.coli* paste (50% *E.coli* paste in sterile M9). Growth was performed at 20°C and the shaking speed was 350 r.p.m. Growth was monitored until most worms were gravid hermaphrodites. The following steps were used to synchronize dense cultures of worms. The *C.elegans* culture were filtered through two layers of Miracloth (Calbiochem) placed over a funnel and rinsed extensively with tap water. The worms were transferred to 50 ml Falcon tubes and bleached by adding 6–8 volumes of fresh bleach solution (20% NaOCl, 2 M NaOH) and stirring in a beaker until complete breakage of the worms (5–7 min). The embryos were pelleted in a clinical centrifuge and washed several times in sterile water. The embryo pellets were then resuspended in complete S medium and transferred into a 2 l fluted flask containing 1 l of complete culture

medium. Synchronized growth was achieved by addition of food once most embryos had hatched and were arrested at the L1 stage. The growth of the culture was then monitored daily until the culture was essentially composed of gravid hermaphrodites (3 days after first feeding).

To prepare nuclear extracts, the embryos were harvested as described above except that they were washed more extensively to remove the bleach. Sterile water was replaced by cold homogenization buffer (HB: 15 mM HEPES pH 7.6, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 44 mM sucrose, 1 mM DTT, 0.5 mM PMSF, 1% aprotinin, 1 mM sodium metabisulfite). The embryo pellets were resuspended in 3 ml HB/g of embryos. The suspension was transferred to a pre-cooled Wheaton stainless steel chamber and homogenized by 10–20 strokes with a stainless steel pestle. Mechanical breakage of the embryos was monitored under a dissecting microscope to ensure that most embryos had been ruptured. The homogenate was filtered through one layer of Miracloth. The homogenization chamber was washed with 2 ml of HB/g of embryos and filtered through the same sheet of Miracloth. The filtrate was then spun for 5 min at 900 r.p.m. in a SS34 rotor at 4°C to pellet debris and unbroken embryos. The supernatant was then transferred to a clean tube and spun for 15 min at 8000 r.p.m. in the SS34 rotor at 4°C. The supernatant was discarded and the sides of the tubes were wiped clean. The nuclei were gently resuspended in 5 ml HB/g of starting embryos by pipeting up and down and dispersed by five strokes with an all glass dounce fitted with a B pestle. The nuclei were spun again for 15 min at 8000 r.p.m. and resuspended in 1 ml nuclei buffer (25 mM HEPES pH 7.6, 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 1% aprotinin, 1 mM sodium metabisulfite) per gram of original embryos. The nuclei were dispersed with an all glass dounce fitted with a B pestle, transferred to Oakridge tubes (Beckman) and 1/10 volume 4 M ammonium sulfate pH 8.0 was added. The tubes were shaken energetically and then placed on ice for 30 min. The tubes were then spun for 1 h at 35 000 r.p.m. at 4°C in a Ti60 rotor. The supernatant was transferred carefully to a clean Oakridge tube and solid ammonium sulfate (0.3 g/ml of supernatant) was added and allowed to dissolve slowly. Once the ammonium sulfate was dissolved, the tubes were left on ice for 30 min before spinning for 15 min at 35 000 r.p.m.. The supernatant was aspirated off and the protein pellet was resuspended in 0.1 ml nuclear dialysis buffer (NDB: 25 mM HEPES pH 7.6, 20 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT) per gram of starting embryos and dialyzed twice for 2 h against NDB at 4°C. The nuclear extract was spun for 5 min at maximum speed in a microfuge to remove precipitated proteins, aliquoted and frozen in liquid nitrogen. The extracts were kept at –80°C where they were stable for at least 18 months. We have prepared extracts from several strains of *C.elegans* and they all showed comparable transcriptional activity.

In vitro transcription

Typical *in vitro* transcription reactions were performed in a final volume of 20 µl. Primer extensions were performed essentially as published (Peterson *et al.*, 1990) and G-less transcription followed the protocol of Gorski *et al.* (1986). The particular final reaction conditions for *C.elegans* were: 200 ng supercoiled DNA template, 17.5 mM HEPES pH 7.6, 40 mM KCl, 7.5% glycerol, 0.625 mM rNTPs, 3 mM $MgCl_2$ and 4 mM spermidine. The amount of nuclear extract used depended on the particular set of experiments and was always added last. The transcriptional activators (UNC-86, MEC-3 and Gal4 fusions) were pre-incubated at room temperature for 10 min with the complete transcription reaction mixture except for the nuclear extract. We have purposely not included any 'control' template DNA lacking MEC-3 and UNC-86 binding sites in our transcription reactions because frequently the presence of such core promoter DNA results in template competition which can influence the levels of activated versus basal transcription. Instead, we have generally attempted to control for the level of activation by performing the reactions multiple times with different preparations of nuclear extracts as well as purified activators. Transcription reactions were performed at 20°C for 30 min and stopped by the addition of 80 µl stop buffer (20 mM Tris pH 7.5, 20 mM EDTA, 0.4 M NaCl, 0.5% SDS), incubated for 15 min at 37°C and extracted once with phenol–chloroform before ethanol precipitation. The radioactive RNAs or cDNAs were run respectively on 6 and 8% polyacrylamide gels. The gels were exposed at –80°C with intensifying screens. Quantitation of transcripts was done using a phosphorimager.

DNA manipulation

Basic DNA manipulations (cloning, probe preparation, DNA sequencing and site-directed mutagenesis) were performed according to Sambrook *et al.* (1989). Footprinting reactions followed previously described protocols (Lichtsteiner *et al.*, 1987), except that each reaction contained typically 1 ng of DNA probe and 1000-fold excess of the non-specific DNA competitor poly(dG–dC)–poly(dG–dC). Proteins and DNA were incubated at room temperature for 20 min and placed on ice for 5 min prior to DNase I digestion which was performed on ice.

The pSL3044 plasmid was constructed as follows: the *mec-3* promoter was excised from pTU#51 (Way and Chalfie, 1989) with *Eco*RI and *Sph*I and cloned in pBS+ cut with the same restriction enzymes to generate pSL3007. pSL3007 was cut with *Eco*RI and *Hin*dIII and the fragment containing the *mec-3* promoter was isolated and its ends were flushed with Klenow enzyme and cloned into pSL2822 cut with *Eco*RI and blunted with Klenow. pSL 2822 contains the minimal *hsp16-2* promoter (TATA box and initiator) cloned in the G-less vector pC_2AT (Sawadogo and Roeder, 1985) at the *Ecl*136II site.

Protein expression and purification

The *unc-86* cDNA was cloned in-frame in the T7 expression vector pET3a (Novagen) by digestion of the cDNA with *Hin*cII and ligation to the vector which was digested with *Bam*HI and blunted using Klenow enzyme. The resulting plasmid pSL3181 was sequenced to ensure the integrity of the construct. The plasmid was transformed into the strain BL21(DE3) pLysS and the resulting clone was grown at 37°C in LB supplemented with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol until the culture reached an OD_{600} of 0.4. The expression of UNC-86 was initiated by the addition of 1 mM IPTG. The culture was then placed at 20°C for 4 h. The induced *E.coli* culture was collected by centrifugation, and the cell pellets were resuspended in 25 mM HEPES pH 7.6, 500 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, 1% aprotinin, 1 mM sodium metabisulfite and frozen in liquid nitrogen. The cells were thawed and sonicated three times for 30 s. The lysate were centrifuged for 30 min at 15 000 r.p.m. in a SS34 rotor at 4°C. The supernatant was collected and diluted to 250 mM NaCl by the addition of one volume of 25 mM HEPES pH 7.6, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, 1% aprotinin, 1 mM sodium metabisulfite and loaded on a DEAE–Sepharose column equilibrated with the same buffer. UNC-86 flowed through the column and was then applied directly to a MonoS column equilibrated with the same buffer. UNC-86 was eluted by a linear NaCl gradient and typically came off between 450 mM and 580 mM. The fractions containing UNC-86 were pooled, dialyzed against the same buffer containing 0.1 M NaCl and loaded on a small DNA affinity column made of the *mec-3* promoter sites CS2 and CS3. The column was washed with NDB 0.3 M NaCl and UNC-86 was eluted with NDB 0.6 M.

Full-length MEC-3 and deletion mutants were expressed as N-terminal FLAG fusion proteins (IBI) in Sf9 cells infected with the appropriate recombinant baculoviruses according to O'Reilly *et al.* (1992). The recombinant proteins were purified from Sf9 cells as follows: after 48 h of infection, the cells were harvested and centrifuged at 900 r.p.m. in 50 ml Falcon tubes. The pellet of cells was resuspended in 0.25 ml of 25 mM HEPES pH 7.6, 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, 10 µM $ZnSO_4$, 0.5 mM PMSF, 1% aprotinin, 1 mM sodium metabisulfite per 1×10^7 cells and sonicated for 3×20 s. The cell lysates were then spun at 15 000 r.p.m. in a SS34 rotor for 20 min at 4°C. The supernatant was discarded and the pellets were resuspended in the same volume of buffer containing 0.5 M NaCl, sonicated and centrifuged as above. The supernatants contained most of the MEC-3 proteins and were used directly for immunopurification using anti-Flag beads (Kodak).

The Gal4 fusion proteins were expressed in Sf9 cells and purified as described above.

Large-scale purification of MEC-3 for the preparation of MEC-3 agarose resin was done as follows. MEC-3 was expressed in *E.coli* as a His–Tag fusion protein using the T7 system. The protein was purified over Ni^{2+}–NTA agarose under denaturing conditions according to the manufacturer's protocol (Qiagen). The purified protein was renatured according to Xue *et al.* (1993) and cross-linked to activated affigel resin (BioRad).

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References

Biggin,M.D. and Tjian,R. (1988) Transcription factors that activate the *Ultrabithorax* promoter in developmentally staged extracts. *Cell*, **53**, 699–711.

Chalfie,M. and Au,M. (1989) Genetic control of differentiation of the *Caenorhabditis elegans* touch receptor neurons. *Nature*, **243**, 1027–1033.

Desplan,C., Theis,J. and O'Farrel,P.H. (1985) The *Drosophila* developmental gene *engrailed* encodes a sequence specific DNA binding activity. *Nature*, **318**, 630–635.

Finney,M. and Ruvkun,G. (1990) The *unc-86* gene product couples cell lineage and cell identity in *C.elegans*. *Cell*, **63**, 895–905.

Finney,M., Horvitz,H.R. and Ruvkun,G. (1988) The *C.elegans* cell lineage and differentiation gene *unc-86* encodes a protein with a homeodomain and extended similarity to transcription factors. *Cell*, **55**, 757–769.

Freyd,G., Kim,S.K. and Horvitz,R. (1990) Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene *lin-11*. *Nature*, **334**, 876–879.

Gorski,K., Carneiro,M. and Schibler,U. (1986) Tissue-specific *in vitro* transcription from the mouse albumin promoter. *Cell*, **47**, 767–776.

Goutte,C. and Johnson,A.D. (1993) Yeast **a**1 and α2 homeodomain proteins form a DNA-binding activity with properties distinct from those of either protein. *J. Mol. Biol.*, **233**, 359–371.

Heiermann,R. and Pongs,O. (1985) *In vitro* transcription with extracts of nuclei of *Drosophila* embryos. *Nucleic Acids Res.*, **13**, 2709–2730.

Hoey,T. and Levine,M. (1988) Divergent homeobox proteins recognize similar DNA sequences in *Drosophila*. *Nature*, **332**, 858–861.

Ingham,P.W. (1991) Segment polarity and cell patterning within the *Drosophila* body segment. *Curr. Opin. Genet. Dev.*, **1**, 417–421.

Lawrence,P.A. and Morata,G. (1994) Homeobox genes, their function in *Drosophila* segmentation and pattern formation. *Cell*, **78**, 181–189.

Lichtsteiner,S., Wuarin,J. and Schibler,U. (1987) The interplay of DNA-binding proteins on the promoter of the mouse albumin gene. *Cell*, **51**, 963–973.

Lin,Y.-S., Carey,M.F., Ptashne,M. and Green,M.R. (1988) GAL4 derivatives function alone and synergistically with mammalian activators *in vitro*. *Cell*, **54**, 659–664.

Nüsslein-Vohlard,C. (1991) Determination of the embryonic axes of *Drosophila*. *Development*, **113 (suppl. 1)**, 1–10.

O'Reilly,D.R., Miller,L.K. and Luckow,V.A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*. W.H.Freeman, New York, NY.

Perrimon,N. and Desplan,C. (1994) Signal transduction in the early *Drosophila* embryo, when genetics meets biochemistry. *Trends Biochem. Sci.*, **19**, 509–513.

Peterson,M.G., Tanese,N., Pugh,B.F. and Tjian,R. (1990) Functional domains and upstream activation properties of cloned human TATA binding protein. *Science*, **248**, 1625–1630.

Pugh,B.F. and Tjian,R. (1990) Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell*, **61**, 1187–1197.

Sadler,I., Crawford,A.W., Michelsen,J.W. and Beckerle,M.C. (1992) Zyxin and cCRP, two interactive LIM domain proteins associated with the cytoskeleton. *J. Cell Biol.*, **119**, 1573–1587.

SambrookJ., Fritsch,E.F. and Maniatis,T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanchez-Garcia,I., Osada,H., Forster,A. and Rabbits,T.H. (1993) The cysteine-rich LIM domains inhibit DNA binding by the associated homeodomain in Isl-1. *EMBO J.*, **12**, 4243–4250.

Sawadogo,M. and Roeder,R.G. (1985) Factors involved in specific transcription by human RNA polymerase II: analysis by a rapid and quantitative *in vitro* assay. *Proc. Natl Acad. Sci. USA*, **82**, 4394–4398.

Schier,A.F. and Gehring,W.J. (1992) Direct homeodomain–DNA interaction in the regulation of the *fushi tarazu* gene. *Nature*, **356**, 804–807.

Soeller,W.C., Poole,S.J. and Kornberg,T. (1988) *In vitro* transcription of the *Drosophila engrailed* gene. *Genes Dev.*, **2**, 68–81.

Sulston,J. and Hodgkin,J. (1988) Methods. In Wood,W.B. (ed.), *The Nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 587–606.

Thali,M., Müller,M.M., DeLorenzi,M., Mathias,P. and Bienz,M. (1988) *Drosophila* homeotic genes encode transcriptional activators similar to OTF-2. *Nature*, **336**, 598–600.

Turner,R. and Tjian,R. (1989) Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos–cJun heterodimers. *Science*, **243**, 1689–1694.

Way,J.C. and Chalfie,M. (1988) *mec-3*, a homeo-box-containing gene that specifies differentiation of the touch receptor neurons in *C.elegans*. *Cell*, **54**, 5–16.

Way,J.C. and Chalfie,M. (1989) The *mec-3* gene of *Caenorhabditis elegans* requires its own product for maintained expression and is expressed in three neuronal cell types. *Genes Dev.*, **3**, 1823–1833.

Way,J.C., Wang,L., Run,J.-Q. and Wang,A. (1991) The *mec-3* gene contains *cis*-acting elements mediating positive and negative regulation in cells produced by asymmetric cell division in *Caenorhabditis elegans*. *Genes Dev.*, **5**, 2199–2211.

Xue,D., Finney,M., Ruvkun,G. and Chalfie,M. (1992) Regulation of the *mec-3* gene by the *C.elegans* homeoproteins UNC-86 and MEC-3. *EMBO J.*, **11**, 4969–4979.

Xue,D., Tu,Y. and Chalfie,M. (1993) Cooperative interactions between the *Caenorhabditis elegans* homeoproteins UNC-86 and MEC-3. *Science*, **261**, 1324–1328.

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