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REPORTER GENE CONSTRUCTS SUGGEST THAT THE *CAENORHABDITIS ELEGANS* AVERMECTIN RECEPTOR β-SUBUNIT IS EXPRESSED SOLELY IN THE PHARYNX

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Summary

Gene promoter/LacZ reporter constructs were made in order to analyse the expression of the β-subunit of the *Caenorhabditis elegans* glutamate-gated Cl^- channel (Glu-Cl) receptor. Southern blot analysis of the *C. elegans* cosmid C35E8 identified a 4 kbp *Eco*RI fragment which contained the 5′ portion of the Glu-Cl β coding sequence together with 5′ flanking sequences. This was subcloned and used as the template for polymerase chain reaction (PCR) amplification of a DNA fragment encoding the first 24 amino acid residues of Glu-Cl β together with 1.4 kbp of 5′ genomic sequence. The fragment was subcloned into the LacZ expression vector pPD22.11 to form a translational reporter fusion. After injection of the construct into worms, six stably transformed lines were established and assayed for β-galactosidase activity. Stained nuclei were observed in the pharyngeal metacorpus in adults and in all larval stages, and stained nuclei were seen in many embryos undergoing morphogenesis. Additional stained nuclei towards the terminal bulb of the pharynx were observed in larval stages. These results provide further evidence that the Glu-Cl receptor mediates the glutamatergic inhibition of pharyngeal muscle *via* the M3 motor neurone and point to inhibition of pharyngeal pumping as a major mode of action for avermectins.

Key words: nematode, *Caenorhabditis elegans*, glutamate-gated Cl^- channel, anthelmintic, reporter gene, avermectin receptor.

Introduction

The avermectins are a group of macrocyclic lactones with exceptional insecticidal and anthelmintic activity (Campbell and Benz, 1984). They are generally believed to exert a paralysing effect on the muscle of target organisms through the opening of Cl^- channels (Martin and Pennington, 1989; Arena *et al.* 1991). Injection of *C. elegans* mRNA into *Xenopus laevis* oocytes results in the expression of a glutamate-gated Cl^- (Glu-Cl) channel that is potentiated by avermectin (Arena *et al.* 1992). The relative efficacy of different avermectin derivatives has been shown to correlate with their potency at this channel (Arena *et al.* 1995), supporting the claim that this represents the major target for avermectins in nematodes. Glutamate-gated Cl^- channels have also been proposed as a target for avermectins in insects and crustaceans (Duce and Scott, 1985; Zufall *et al.* 1989).

Cully *et al.* (1994) have expression-cloned an avermectin receptor from *C. elegans*. Two subunits were isolated, a glutamate-sensitive β-subunit and an avermectin-sensitive α-subunit which, when co-expressed in *Xenopus* oocytes, formed avermectin-sensitive glutamate-gated Cl^- channels. In sequence comparisons, both subunits show highest sequence identity to previously cloned mammalian and invertebrate $GABA_A$ and glycine receptors. In vertebrates, γ-aminobutyric acid (GABA) and glycine are the major inhibitory neurotransmitters, mediating their effects *via* Cl^- channels. GABA is also a major inhibitory neurotransmitter in invertebrates, and GABA receptor subunits have been isolated from *Drosophila melanogaster* (ffrench-Constant *et al.* 1991; Henderson *et al.* 1993; Harvey *et al.* 1994), *Aedes aegypti* (Thompson *et al.* 1993) and the freshwater snail *Lymnaea stagnalis* (Harvey *et al.* 1991; Hutton *et al.* 1993). On the basis of their primary sequence, these receptors have the same general structure as the vertebrate subunits. In nematodes, GABA is the major neuromuscular inhibitory transmitter (Del Castillo *et al.* 1964*a*,*b*), and the pharmacology of the receptor resembles that of vertebrate $GABA_A$ receptors, although there are important differences, especially regarding the antagonist profile and modulatory binding sites (Holden-Dye *et al.* 1989; Duittoz and Martin, 1991). A number of previous studies have shown modulatory effects on nematode muscle GABA receptors by avermectin (Holden-Dye *et al.* 1988; Holden-Dye and Walker, 1990). More recently, however, the focus of attention has turned to the related class of avermectin-sensitive glutamate receptors discovered in *C. elegans*.

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Although a putative avermectin receptor has been cloned from *C. elegans*, the mode of action of avermectins is still unknown. Pharmacologically relevant concentrations of avermectin have no visible effect on motility in susceptible nematode species, suggesting that muscle GABA receptors are not the target for avermectin action (Geary *et al.* 1992). However, pharyngeal pumping in *C. elegans* is extremely sensitive to avermectin (Avery and Horvitz, 1990), and avermectin concentrations between 0.1 and 1 nmol l^{-1} paralyse the pharynx of *Haemonchus contortus* (Geary *et al.* 1993) and reduce feeding in *Trichostrongylus colubriformis* (Bottjer and Bone, 1985). Gill *et al.* (1995) have looked at the effects of avermectins on the inhibition of larval development in *H. contortus*. Concentrations of avermectin greater than 30 nmol l^{-1} were required for the effective paralysis of L1 larvae soon after hatching, but much lower concentrations (approximately 1 nmol l^{-1}) were sufficient to inhibit larval development to the L3 stage, most probably through the inhibition of pharyngeal pumping and feeding. These results clearly demonstrate that pharyngeal muscle is much more sensitive to the effects of avermectin than is body muscle.

We have employed the DNA transformation methods developed by Fire (1986) and Mello *et al.* (1991) to analyse the expression pattern of the *C. elegans* Glu-Cl β-subunit. These methods involve the construction of reporter fusions where the promoter elements for the gene of interest are linked to a reporter gene such as the *Escherichia coli lacZ* gene, encoding β-galactosidase (Fire *et al.* 1990). Transgenic nematodes are generated by micro-injection of the reporter construct into the adult gonad followed by the selection of transformed F_1 progeny and the establishment of transgenic lines. Animals are fixed and incubated with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) to localise β-galactosidase activity. These methods allow for the temporal and spatial characterisation of gene expression patterns in *C. elegans*.

Materials and methods

The N2 strain of *Caenorhabditis elegans* was used throughout the work (Brenner, 1974). Nematodes were routinely cultured on NGM agar plates seeded with OP50, a uracil-requiring mutant of *Escherichia coli* (Sulston and Brenner, 1974). Plates were incubated at 20 °C and required subculturing approximately every 7 days.

Southern analysis

Restriction digests of cosmid DNA were electrophoresed through a 1 % agarose gel and transferred onto Hybond-N nylon membrane (Amersham). Radiolabelled cDNA probes were prepared by random-primed labelling using random hexameric oligonucleotides and [^{32}P]dCTP in the presence of T_7 DNA polymerase. Filters were hybridised in Rapid-hyb buffer (Amersham) at 68 °C for 3 h. Filters were washed once in 50 ml of 2×SSC (0.15 mol l^{-1} NaCl, 0.015 mol l^{-1} sodium citrate), 0.1 % SDS for 20 min at room temperature and twice in 0.1×SSC, 0.1 % SDS for 15 min at 65 °C, air-dried and exposed to X-ray film overnight.

C. elegans *micro-injection*

Micro-injections of adult hermaphrodites were performed as described by Mello and Fire (1995) using a Zeiss Axiovert 10 microscope and a Narashige model 202 micromanipulator. Reporter constructs together with the plasmid pRF4 (100–200 μg ml^{-1}) were injected into the cytoplasmic syncytium of the gonad. pRF4 carries the dominant mutation *su1006* in the *rol 6* collagen gene, which causes animals to roll and move in circles, acting as a marker for transformation. Rolling F_1 progeny were picked and the subsequent F_2 generations surveyed for transformants. Progeny showing a high frequency of transmission (10–50 %) were chosen for the establishment of transgenic lines.

β-Galactosidase assay

Animals were fixed and assayed for activity using a modification of the protocol described by Fire (1992). Briefly, nematodes were washed off agar plates using water, placed onto a slide and overlaid with a coverslip. The slide was transferred to a metal plate on dry ice for 5 min, after which the coverslip was levered off and the slide plunged into methanol at −20 °C for 5 min. The slide was transferred to acetone at −20 °C for a further 5 min. Slides were air-dried, 25 μl of staining mix (0.024 % X-gal in 0.2 mol l^{-1} sodium phosphate, 1 mmol l^{-1} magnesium chloride, 5 mmol l^{-1} potassium ferricyanide and 5 mmol l^{-1} potassium ferrocyanide, 0.04 % SDS, 0.075 mg ml^{-1} kanamycin sulphate) was added and a coverslip overlaid. Slides were incubated at 37 °C until staining was clearly visible (3–24 h).

Synchronous C. elegans *cultures*

Synchronous cultures were established as described by Sulston and Hodgkin (1988). Eggs were obtained by digesting populations containing many gravid worms with alkaline hypochlorite (1 % sodium hypochlorite, 0.25 mol l^{-1} KOH). After centrifugation, the pelleted eggs were transferred to S medium (0.1 mol l^{-1} NaCl, 0.05 mol l^{-1} potassium phosphate, pH 6.0, 5 mg l^{-1} cholesterol, 0.01 mmol l^{-1} potassium citrate, pH 6.0, 0.03 mmol l^{-1} $CaCl_2$, 0.03 mmol l^{-1} $MgSO_4$, 50 μmol l^{-1} Na_2 EDTA, 250 μmol l^{-1} $FeSO_4{\cdot}7H_2O$, 10 μmol l^{-1} $MnCl_2{\cdot}4H_2O$, 10 μmol l^{-1} $ZnSO_4{\cdot}7H_2O$, 1 μmol l^{-1} $CuSO_4{\cdot}5H_2O$) without bacteria and allowed to hatch overnight at 20 °C. The subsequent L1 larvae were then transferred to NGM plates with bacteria, allowing the development of a synchronised culture.

Results

Reporter constructs

The localisation of the Glu-Cl β-subunit gene to the *C. elegans* cosmid C35E8, has been described previously (Laughton *et al.* 1995). C35E8 was digested with a variety of restriction enzymes and blotted onto duplicate Hybond-N

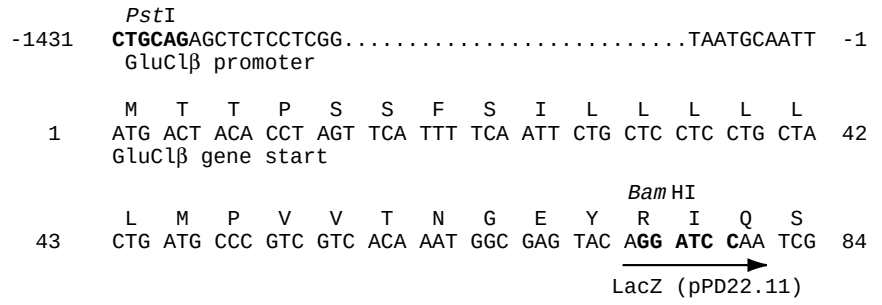

Fig. 1. Glu-Cl β-gene promoter/pPD22.11 LacZ reporter construct. The genomic DNA fragment was inserted into the pPD22.11 expression vector on *Pst*I and *Bam*HI sites (shown in bold type). Nucleotides are numbered beginning at the start methionine of the Glu-Cl β-gene. The translational fusion with LacZ is marked by an arrow.

membranes (Amersham). Each filter was then hybridised with a cDNA probe corresponding to either the N-terminal 154 amino acid residues or the C-terminal 185 amino acid residues of the Glu-Cl β-subunit. Hybridising bands in each filter were compared and a 4 kbp *Eco*RI fragment was identified which contained the N-terminal but not the C-terminal region of the Glu-Cl β-subunit gene. This *Eco*RI fragment was subcloned into pBluescript SK^+ for DNA sequencing, and the construct was used as a template for PCR. A forward primer incorporating a *Pst*I site (5′-TGTGTTCTGCAGAGCTCTCCTCGGAGCCCA-3′) was used with a reverse primer incorporating a *Bam*HI site (5′-CCGATTGGATCCTGTACTCGCCAT-3′) to amplify a product consisting of 1.4 kb of 5′ sequence together with sequence encoding the first 24 amino acid residues of the Glu-Cl β-subunit. The PCR product was subcloned into the expression vector pPD22.11 (Fire *et al.* 1990) to form a translational fusion with the *lacZ* gene (Fig. 1).

Analysis of Glu-Cl β-subunit expression in transgenic C. elegans

Following micro-injections of the Glu-Cl β reporter construct, six heritably transformed *C. elegans* lines were established and assayed for β-galactosidase activity. The pPD22.11 fusion vector contains the nuclear localisation signal from the SV40 T-antigen, which targets β-galactosidase to the nucleus of cells expressing this construct. Adults from all six transformed lines showed consistent staining of nuclei within the pharynx (Fig. 2A), and no staining was observed in other organs. From their position and the shape of the corresponding cells, six of these stained nuclei were identified as those of the m4 pharyngeal muscle cells in the metacorpus (Fig. 3). In adults, no nuclei outside the metacorpus consistently stained for X-gal, although very faint staining of nuclei in the terminal bulb was sometimes seen (indicated by the small arrowhead in Fig. 2A).

Developmental expression of Glu-Cl β-subunit

Synchronous cultures were set up for two of the transformed lines to analyse the developmental expression of the Glu-Cl β-subunit. All the larval stages showed staining of pharyngeal nuclei (Fig. 2B,C), as seen in the adults, and stained nuclei were also observed in many eggs undergoing morphogenesis (Fig. 2D). In addition to the metacorpus staining, larvae (L1–L3) also exhibited staining of nuclei towards the terminal bulb of the pharynx (see Fig. 2B–D). Exact identification of these nuclei in the terminal bulb proved difficult owing to the diffuse nature of the staining observed at the earlier stages of development. This staining was most pronounced in eggs undergoing the later stages of morphogenesis and generally decreased throughout development until it was completely absent or barely visible in adults (Fig. 2A).

Discussion

These LacZ reporter experiments have shown that the Glu-Cl β-subunit promoter is active in pharyngeal cells of *C. elegans*. Adults and all larval stages appear to express this subunit in the three m4 pharyngeal muscle cells, which comprise the metacorpus, and these are the only nuclei that consistently stained in adults. These muscles are required for pharyngeal pumping/feeding, and their relaxation is triggered *via* the fast inhibitory motor neurone M3 (Raizen and Avery, 1994). Although reporter gene fusions do not always faithfully reflect the expression patterns of the intact gene, perhaps because of the absence of enhancer elements located downstream or within introns, there are additional physiological data to support the conclusion that this subunit is expressed on pharyngeal muscle cells. Raizen and Avery (1994) have shown that the inhibitory M3 motor neurone is unlikely to be GABAergic and have suggested more recently that it is glutamatergic (L. Avery, M. W. Davis, W. Denk, J. Dent and G. Hess, personal communication) and, given that pharyngeal pumping is extremely sensitive to the avermectins (Avery and Horvitz, 1990), it seems likely that transmission is mediated *via* the glutamate-gated avermectin receptor cloned by Cully *et al.* (1994). Martin (1996) has made two-microelectrode current-clamp recordings from pharyngeal muscle preparations of *Ascaris suum* and has demonstrated the presence of glutamate-gated Cl^- channels on the pharyngeal muscle of this important parasitic nematode. In addition, he demonstrated a dose-dependent potentiation of the glutamate response by the avermectin analogue milbemycin D.

The data reported here thus provide further evidence that an inhibitory glutamate-gated Cl^- channel is expressed on nematode pharyngeal muscle. This channel, the ‘avermectin’ receptor, may be the primary target of the avermectins, and the effects of the drug may be due to the paralysis of pharyngeal muscle. How does this account for the anthelmintic activity? Paralysis of the pharynx will prevent nematodes from feeding,

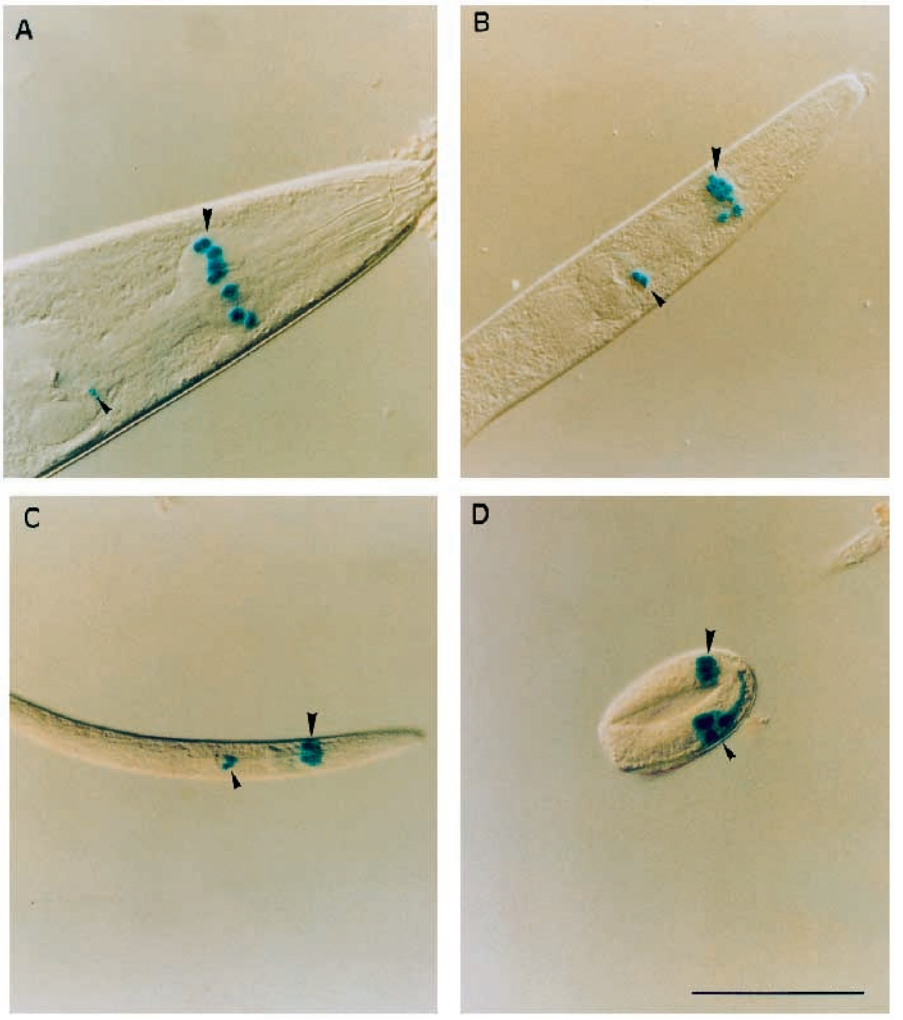

Fig. 2. Localisation of β-galactosidase activity in the pharynx of transgenic *Caenorhabditis elegans* during development. Staining of the pharyngeal metacorpus nuclei in adults (A), L3 larvae (B), L1 larvae (C) and eggs (D) is marked by the large arrowheads. Staining of additional nuclei within the terminal bulb of the pharynx is highlighted by the small arrowheads. Scale bar, 50 μm.

leading to arrested development, starvation and eventual death. The inhibition of larval development has been demonstrated *in vitro* for *Haemonchus contortus*, where concentrations of avermectin as low as 1 nmol l^{-1} were sufficient to prevent development of larvae to the L3 stage (Gill *et al.* 1995). Nevertheless, it remains unclear how this could account for the rapid expulsion of adult parasites from the treated host. Furthermore, there is evidence that many gastrointestinal nematodes are able to absorb nutrients through the cuticle as well as by ingestion (Ho *et al.* 1990; Geary *et al.* 1993). It is possible that avermectins kill the parasite through the interruption of other vital functions served by the pharynx, for example the regulation of turgor pressure. Adult filarial nematodes depend on the transcuticular absorption of nutrients (Howells, 1980). Here, the lack of avermectin anthelmintic activity on macrofilariae fits with the proposed site of action and the interruption of oral ingestion. The localisation of avermectin receptors in parasitic nematode species will be necessary to verify the results obtained from *C. elegans*.

Avermectins may act at additional sites in nematodes. Our observations on the developmental expression of the Glu-Cl β-subunit suggest that there may be some developmentally regulated expression of this subunit within the pharynx. One of these sites is probably the m5 muscle cell which forms the isthmus of the pharynx, nuclei of which are occasionally weakly stained in adults. Interestingly, Albertson and Thomson (1976) noted that the glutamatergic M3 motor neurone which innervates m4 pharyngeal muscle cells also sends an occasional synapse to m5 muscle cells. Although the pattern of expression in adults is largely limited to the m4 cells, it is clear the reporter gene is active in additional pharyngeal nuclei at earlier stages of development, especially in the terminal bulb, and that the pattern of staining becomes simpler through development. The diffuse nature of this larval staining makes it difficult to identify the specific nuclei concerned unequivocally, but it is likely that additional pharyngeal cells are expressing the subunit at earlier stages of development and possible that the effects of avermectins on larval development may be related to this expression.

Although the glutamate-sensitive β-subunit of the avermectin receptor appears to be expressed exclusively in the pharynx, we have not localised the avermectin-sensitive α-subunit. This subunit may co-assemble with other subunits to form additional avermectin receptors that are expressed at other sites. For example, the α_6-subunit of the mammalian brain $GABA_A$ receptor exists as distinct $\alpha_6\gamma$ and $\alpha_6\delta$ receptor

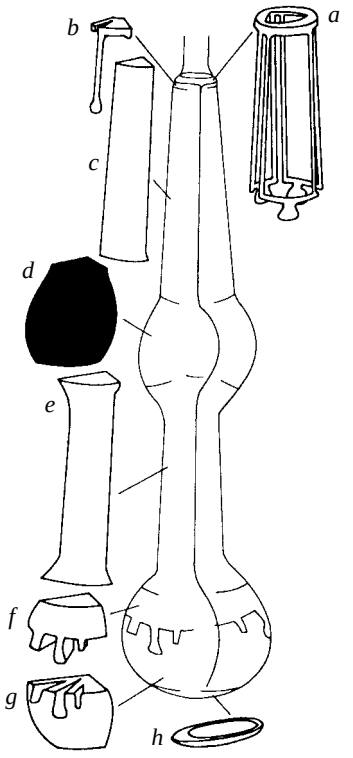

Fig. 3. *Caenorhabditis elegans* pharyngeal muscle cells. The eight muscle layers are labelled *a–h*, and a single cell is pictured for each layer. Muscle layers m3 (*c*), m4 (*d*) and m5 (*e*) are composed of three wedge-shaped cells, each cell containing two nuclei. Muscles m4 (shaded black) are more spherical than the others, forming the well-developed metacorpus. The three m5 muscle cells form the isthmus, and their associated nuclei lie at the posterior of the cell. In the terminal bulb, three T-shaped cells (*f*) slot into a further set of three cells (*g*). A saucer-shaped cell (*h*) lines the posterior of the pharynx. Reproduced and modified, with permission, from Fig. 21 of Albertson and Thomson (1976).

populations in the cerebellum (Quirk *et al.* 1994). A further complication is the growing evidence that there may be multiple inhibitory glutamate receptors composed of a heterogeneous family of subunits. We have cloned two additional subunits from *C. elegans* (Cegbr2 and Cegbr3) which show a high level of sequence identity to the avermectin receptor subunits (D. L. Laughton, G. G. Lunt and A. J. Wolstenholme, manuscript in preparation) and J. Dent and L. Avery (personal communication) have recently cloned a second α-subunit from *C. elegans* (Glu-Cl α2) which has 85 % identity at the amino-acid level to Glu-Cl α. Similar LacZ reporter studies for the α-subunits and the use of subunit-specific antibodies may help to elucidate further the mode of action of avermectins.

Taken together, these data provide strong evidence that the pharynx is a major target for avermectins. However, the exact nature and composition of the avermectin receptor(s) remains to be determined.

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