C. ELEGANS GENE M03F4.3 IS A D2-LIKE DOPAMINE RECEPTOR

by

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ABSTRACT

In *C. elegans* there are over 1000 putative G protein-coupled receptor (GPCR) genes, of which approximately 18 are predicted to code for rhodopsin-like GPCRs. Heterotrimeric GTP-binding proteins mediate the response to a wide variety of neurotransmitters and hormones, all of which bind to GPCRs. I have chosen to work on M03F4.3, which is a candidate rhodopsin-like GPCR. Although M03F4.3 is believed to code for a rhodopsin-like GPCR, its functions are not yet clear. To investigate its functions, a region upstream of M03F4.3 putatively containing the complete promoter has been stitched to GFP-encoding DNA and injected into *C. elegans* hermaphrodites to create transgenic animals. Thus, the expression patterns can be observed with GFP fluorescence. Expression was observed in anterior deirid and cephalic sensilla, vulva, intestine, rectum gland cells, and one posterior cell. The anterior deirid sensilla and the cephalic sensilla comprise two ADE neurons and two CEP neurons, respectively, which are known to respond to dopamine. In addition, a BLASTp search has revealed that *C. elegans* genes K09G1.4 (*dop-2*), F15A8.5 (*dop-1*), C02D4.2 (*ser-2*), K02F2.6 (*ser-3*), Y22D7AR.13 (*ser-4*), and C09B7.1 (*ser-7*) share high similarities, based on amino acid sequence, with M03F4.3. The expression pattern of K09G1.4 is similar to M03F4.3, which makes M03F4.3 a good candidate for expression of dopamine receptor. Furthermore, the regulatory elements that govern the expression of M03F4.3 in were deduced by constructing a series of deletions in the promoter region to GFP and then injecting into *C. elegans* hermaphrodites. In addition, experiments with M03F4.3 knockout and *dop-1*, *dop-2*, *ser-2*, *ser-3*, or *ser-7* RNAi constructs have been conducted to determine any changes in mating behavior, locomotion and morphology of both

hermaphrodites and males. No changes, however, have been detected. Moreover, phylogenetic tree has been assembled to determine which of two subfamilies, D1-like and D2-like dopamine receptors, that M03F4.3 belongs to. The gene clustered with D2-like dopamine receptor encoding genes in different organisms. These findings suggest that M03F4.3 is a likely candidate to code for a D2-like dopamine receptor.

To my parents, Zouya and Sarkis Ohanian, with my deepest love and gratitude for their love, faith and support.

ACKNOWLEDGEMENTS

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1 INTRODUCTION

1.1 General features of *Caenorhabditis elegans* as a model system

The free-living soil nematode *Caenorhabditis elegans* is an attractive model system to study many biological processes (Brenner *et al.* 1974). *C. elegans* is amenable to a variety of genetic, cellular, and molecular analysis due to its rapid life cycle (~3 ½ days at 20°C), small size (1 mm in length as an adult), large brood sizes (over 300 progeny per hermaphrodite), and ease of maintenance in the laboratory (easily cultured on agar plates seeded with *Escherichia coli* as a food source). The transparency of the animal allows visualization of fine anatomical details and single cells (<1000 cells), including the 302-cell hermaphrodite nervous system, with a compound microscope equipped with Nomarski DIC optics. Transgenic worms can be created by direct microinjection of expression constructs, which comprise plasmid or cosmid DNA stitched to green fluorescent protein (GFP). The tagging of GFP allows for examination of neuronal morphology and observation of protein expression patterns in the living nervous system (under fluorescent microscope). Although *C. elegans* has a very simple nervous system compared to mammalian standards, it contains genes that encode most of the known molecular components of mammalian brains. That is, the *C. elegans* nervous system contains many of the known signaling components and neurotransmitter systems, such as membrane and vesicular neurotransmitter-specific transporters and G protein-coupled receptors (GPCRs), found in the mammalian nervous system. Moreover, *C. elegans* is a self-fertilizing hermaphrodite with the ability to cross-fertilize with males. These

characteristics allow for rapid protocols to identify candidate loci with genetic screens. In addition, generation of gene knockouts and RNA-mediated interference (RNAi) technology can be used to assess loss-of-function mutant phenotypes in most cell types. RNAi is used as a reverse-genetics tool for determining functions of specific genes, which is achieved by inhibiting the activity of a corresponding gene by the introduction of double-stranded RNA (dsRNA) (Fire *et al.* 1998). This technique rapidly produces gene-specific loss-of-function or hypomorphic phenotypes. It is important to note that, however, RNAi may not be an efficient technique for two reasons. First, RNAi may be ineffective against some targeted genes. RNAi does not accurately phenocopy the null phenotype of all genes, such as the ones involved in neuronal function, and may result in either partial or no loss of function; second, either subtle or conditional phenotypes will not be detected (Fraser *et al.* 2000). In these cases, generation of gene knockout strains, which can be provided by the *C. elegans* Gene Knockout Consortium, may be a more reliable method. Furthermore, the recent completion of *C. elegans* genomic sequence (100.3 Megabase pairs (Mbp)) provides researchers in diverse fields with a rich resource of genomic and bioinformatic data (*C. elegans* Sequencing Consortium, 1998). The developmental fates of the 959 somatic cells of the hermaphrodite have been mapped, and the locations and connectivity of the 302 neurons have been described through serial electron micrograph sections (Albertson & Thomson 1976; White *et al.* 1986). These advantages have proven *C. elegans* as an important tool for revealing and characterizing genes, and for providing insights into mammalian genes.

1.2 The structure of nervous system

C. elegans' hermaphroditic nervous system comprises 302 neurons, which play significant roles in a number of key processes including egg-laying, defecation, locomotion, and pharyngeal pumping (Riddle *et al.* 1997). In *C. elegans* hermaphrodites, these neurons have been reconstructed from serial section electron micrographs to define the morphology of each neuron, its chemical synapses, and its gap junctions. The nervous system contains approximately 2000 neuromuscular junctions, 5000 chemical synapses between neurons, and 700 gap junctions. These neurons, divided into 118 classes with 1 to 13 members in each class, are conventionally classified as sensory neurons, interneurons, or motor neurons (Bargmann & Kaplan 1998). Sensory neurons have sensory endings that sense environmental alterations and are presynaptic to other neurons. Interneurons receive input from and send output to other neurons. They are named for all neurons that cannot be classified as either sensory neurons or motor neurons. Most interneurons are found concentrated in the head and tail ganglia. Some, however, have long axons that span from the head to the tail of the worm. Most sensory neurons and interneurons are bilaterally symmetrical on the left and the right sides of the worm. About half of the 302 neurons, including most sensory neurons, are found in the head around the central neuropil called the nerve ring. The pharyngeal nervous system in *C. elegans* contains 20 neurons and is connected via a single pair of interneurons to the somatic nervous system (Riddle *et al.* 1997).

1.3 G-protein-coupled receptors (GPCRs) and G-protein signaling pathways

In *C. elegans,* heterotrimeric guanine nucleotide (GTP)-binding proteins (G-proteins) mediate the physiological responses to neurotransmitters (i.e., serotonin, dopamine, octopamine), growth factors and hormones, all of which bind to GPCRs (Dohlman et al. 1991, Gilman 1995). GPCRs act as guanine-nucleotide exchange factors for the heterotrimeric G-proteins, which consist of three subunits (α, β, and γ). In the absence of agonist, α subunits are bound to GDP and remain tightly associated with βγ. The activated GPCR, following the addition of agonist, induces a conformational change in the associated G-protein α-subunit leading to the release of GTP followed by binding of GDP. The liberated α-GTP and βγ species propagate the signal by regulating downstream effectors (i.e., adenylyl cyclase, phospholipase C) that can regulate the activities of ion channels. The hydrolysis of GTP terminates the signal and leads to the reformation of the heterotrimer.

Over 1000 putative GPCR-encoding genes have been revealed in *C. elegans*' genome. GPCRs constitute vast families that encompass wide range of functions in fungi, plants, and the animal kingdom. Although they do not share any overall sequence homology, these polytopic membrane proteins share a common structure with 7 transmembrane (7-TM) segments. The 7-TM segments contain seven α-helical hydrophobic amino acid domains connected by alternating intracellular and extracellular peptide loops, with the amino (N) terminus located on the extracellular side and the carboxy (C) terminus on the intracellular side. Although they exhibit diversity at the sequence level, GPCRs can be divided into three major subfamilies: rhodopsin/β2

adrenergic receptors, glucagons/VIP/calcitonin receptor-like, and metabotropic neurotransmitter calcium receptors (Figure 1, as shown in Gether 2000). Of the 1000 putative GPCR genes, about 18 are predicted to code for rhodopsin-like GPCRs in family A in the *C. elegans* genome. M03F4.3 is one of the candidates.

1.4 Rhodopsin-like GPCRs

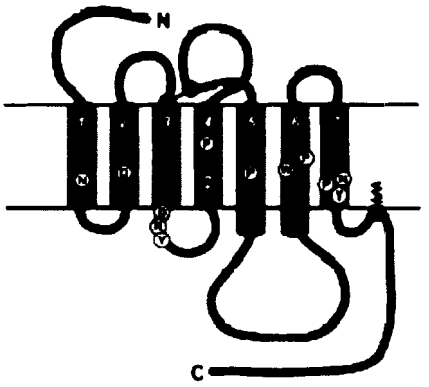
Family A of rhodopsin/β2-adrenergic receptor-like receptors is the largest and the most studied. The receptors of family A are subcategorized further into six different subgroups. For instance, biogenic amine receptors (i.e., serotonin, dopamine, adrenergic), growth hormone secretagogues receptors, and olfactory receptors fall into this category. The homology within the receptors in family A is restricted to highly conserved key residues as indicated in Figure 1, therefore suggesting that the key residues have essential roles for the structural and/or functional integrity of the receptors. For instance, DRY motif is found conserved at the cytoplasmic side of TM segment 3, whereas it is absent in both families B and C.

Among the 18 predicted rhodopsin-like GPCR-encoding genes in the *C. elegans*' genome, M03F4.3 is a candidate. M03F4.3 is located on the X chromosome and produces two different isoforms, M03F4.3a and M03F4.3b. M03F4.3a contains 11 exons spanning 7282 base pairs (bp) of genomic DNA, and codes for a protein of 564 amino acids (aa) (Figure 2, as illustrated at http://www.wormbase.org/db/gene/gene?name=M03F4.3). M03F4.3b, on the other hand, has 11 exons, with the length of 4803bp of genomic DNA, and codes for 592aa protein (Figure 2). The isoforms differ in the location and the size of Exon 1. Exon 1 in M03F4.3a is simply a start codon that resides 3707bp upstream of Exon 2. Meanwhile,

Exon 1 in M03F4.3b is 87bp long and resides 1411 upstream of Exon 2. M03F4.3 exhibits structure that resembles 7-TM with conserved DRY motif between TM segments 3 and 4, WXPF motif on TM segment 6, and WXXY and NPXXY motifs on TM segment 7 (Figure 3). These motifs are conserved across receptors in family A, whereas they are absent in families B and C. Although M03F4.3 is a candidate for family A receptor, its functions remain unknown. In this study, I investigated the possible roles of M03F4.3 as a rhodopsin-like GPCR. To achieve this, a full promoter region of 3700bp upstream of M04F4.3, and downstream of K05B2.5 (*pes-22*) on X chromosome, has been stitched to DNA encoding a green fluorescent protein (GFP), and thus injected into *C. elegans* hermaphrodites to create transgenic worms. To deduce regulatory elements for transcription of the gene in expressed areas, I created and thus constructed a series of deletions in the promoter upstream of the M03F4.3 with GFP. These constructs were subsequently injected into the gonads of *C. elegans* hermaphrodites. In addition, a BLASTp search of the M03F4.3 has been performed; a series of RNA-mediated interference (RNAi) and knockout (KO) has been conducted to determine any phenotypic changes.

Figure 1: GPCRs can be divided into three major subfamilies.

Gether 2000. (Adapted by permission of Ulrik Gether). The receptors in each family are characterized by a several highly conserved key residues, as indicated by black letter in white circles. Most family A receptors comprise a disulfide bridge that connects the second (ECL2) and the third extracellular loop (ECL3) (white letters in black circles). Family B receptors have a long amino terminus containing several cysteines forming a network of disulfide bridges. Family C receptors are characterized by amino terminus with the length of ~600 amino acids. In addition, DRY motif is conserved in family A receptors, whereas it is absent in families B and C.



Family A. Rhodopsin/β2 adrenergic receptor-like

Biogenic amine receptors (adrenergic, serotonin, dopamine, muscarinic, histamine)

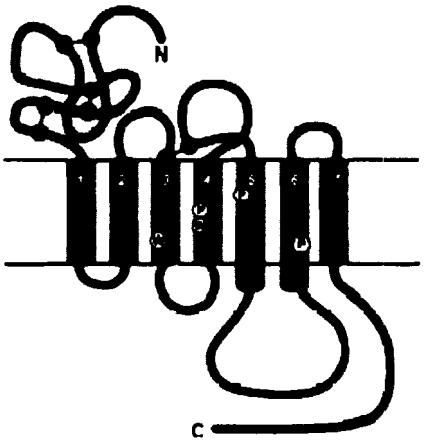
CCK, endothelin, tachykinin, neuropeptide Y, TRH, neurotensin, bombesin, and growth hormone secretagogues receptors plus vertebrate opsins

Invertebrate opsins and bradykinin receptors

Adenosine, cannabinoid, melanocortin, and olfactory receptors.

Chemokine, fMLP, C5A, GNRH, eicosanoid, leukotriene, FSH, LH, TSH, fMLP, galanin, nucleotide, opioid, oxytocin, vasopressin, somatostatin, and protease-activated receptors plus others.

Melatonin receptors and other non-classified



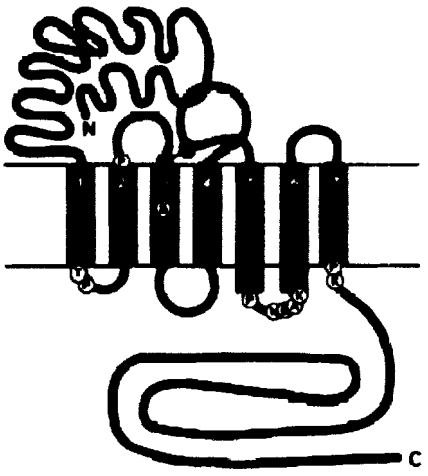
Family B. Glucagon/VIP/Calcitonin receptor-like

Calcitonin, CGRP and CRF receptors

PTH and PTHrP receptors

Glucagon, glucagon-like peptide, GIP, GHRH, PACAP, VIP, and secretin receptors

Latrotoxin



Family C. Metabotropic neurotransmitter/ Calcium receptors

Metabotropic glutamate receptors

Metabotropic GABA receptors

Calcium receptors

Vomeronasal pheromone receptors

Taste receptors

Figure 2: Gene models of M03F4.3.

WormBase web site, www.wormbase.org, release WS128, date 2 August 2004.

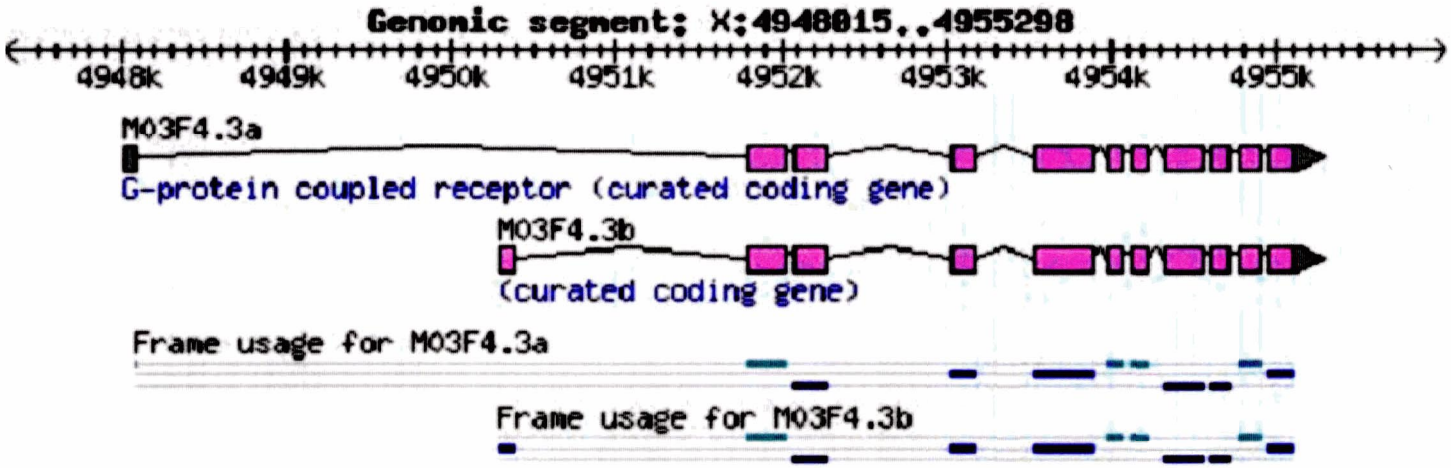
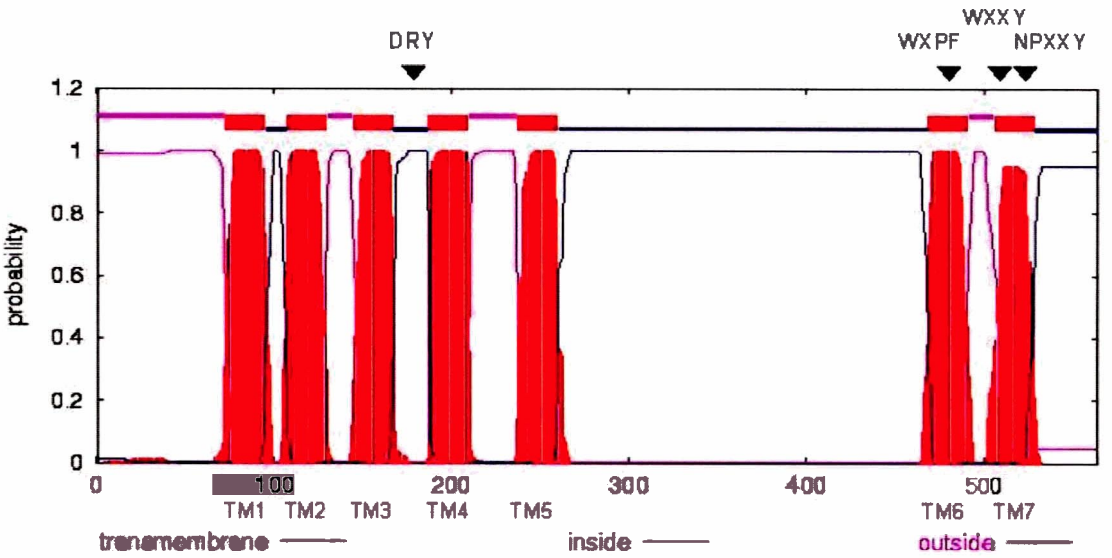

Genomic segment: X:4948015..4955298
4948k
4949k
4950k
4951k
4952k
4953k
4954k
4955k
M03F4.3a
G-protein coupled receptor (curated coding gene)
M03F4.3b
(curated coding gene)
Frame usage for M03F4.3a
Frame usage for M03F4.3b

Figure 3: Transmembrane prediction of M03F4.3 using a hidden Markov model (TMHMM) (based on Krogh *et al* 2001).

M03F4.3 contains DRY, NPXXY, WXPF and WXXY motifs, which are conserved in rhodopsin-like GPCRs. The WXPF and WXXY motifs are thought to be involved in biogenic amine binding (Roth *et al.* 1997), the DRY motif in receptor activation and the NPXXY in receptor desensitization/internalization (Gether 2000).


DRY
WXXY
WXPF
NPXXY
1.2
1
0.8
0.6
0.4
0.2
0
probability
0
100
200
300
400
500
TM1
TM2
TM3
TM4
TM5
TM6
TM7
transmembrane
inside
outside

2 MATERIALS AND METHODS

2.1 Strains and maintenance of *C. elegans*

Strains were maintained on a nutrient growth media (NGM) (0.3% NaCl, 0.25% peptone, 1.7% agar, 5 mg/ml cholesterol, 1 mM CaCl2, 1 mM MgSO4, 25 mM KH2PO4 pH6) plates seeded with *E. coli* OP50 at 15°C or 20°C (Brenner 1974). The following strains were used in this work.

N2 Bristol (wild-type), *dpy-5(e907)*, VC125: M03F4.3*(ok325)*, ASO1: [*M03F4.3p(2047)::gfp*], ASO2: [*M03F4.3p(1447)::gfp*], ASO3: [*M03F4.3p(947)::gfp*], ASO4: [*M03F4.3p(447)::gfp*], ASO5: [*M03F4.3p(323)::gfp*], and ASO6: [*M03F4.3p(200)::gfp*].

2.2 DNA techniques:

2.2.1 Agarose gel electrophoresis

Agarose gels were prepared by adding agarose powder to TBE buffer (2.5 mM NaOH, 89.2 mM Tris base, 89 mM boric acid, 2mM EDTA) (1% agarose gel: 1 gram agarose powder to 100 mL TBE buffer). The solution was heated to boiling to dissolve the agarose. Ethidium bromide was subsequently added. Concentrated loading dye (bromophenol blue) was added to the DNA sample (ratio (bromophenol blue:DNA sample) = 1µl:6µl).

2.2.2 *Restriction analysis and DNA extraction from agarose gels*

DNA fragments were generated by restriction digest with restriction enzymes obtained from New England Biolabs and Invitrogen. Digested DNA samples were analyzed on agarose gels and fragments of interest were eluted using the Gel Extraction Kit (Qiagen, Inc).

2.2.3 *Ligation of endonuclease digested DNA fragments*

For cloning purposes, endonuclease-digested DNA was ligated using T4 DNA ligase (Invitrogen). Ligations were performed based on standard protocols (Ausubel *et al.*, 1993).

2.2.4 *Transformation of DNA into E. coli*

Purified plasmids and ligation reactions were transformed into CaC12 treated competent *E. coli* DH5α cells according to standard protocols (Asubel *et al.*, 1993).

2.2.5 *Plasmid extraction from transformation of E. coli*

Plasmid extraction from *E.coli* cells was performed using the Miniprep Kit (Qiagen, Inc.).

2.2.6 *Polymerase chain reaction (PCR)*

PCR was performed using the Expand Long Template PCR System using their buffer #2.

2.2.7 *Reporter gene constructs*

gfp fusion constructs were constructed using a PCR fusion protocol described in (Hobert 2002). The PCR products were fused to the polylinker of the *gfp* vector

pPD95.75. All constructs were injected into the gonads of *dpy-5(e907)* hermaphrodites at an estimated concentration of 50 ng/µl with *dpy-5*(+) plasmid (*pCeh-361*) (kindly provided by C. Thacker and A. Rose) as an injection marker. Transgenic progeny that appeared wild-type, after getting the array which carries *gfp* fusion constructs and dyp-5(+) plasmid (both in numerous copies in the form of a concatamer), were picked and viewed with Zeiss compound microscope with FITC filters for immunofluorescence.

2.2.8 RNA-mediated interference (RNAi) experiments

RNAi-through-feeding experiments were performed as previously described (Kamath *et. al* 2000). Wild-type and VC125 animals at L4 stage were placed on RNAi-inducing plates and were allowed to lay progeny. Adults were removed or transferred to new RNAi-producing plates. F1 and F3 progeny grown on RNAi plates were scored for phenotypic changes.

2.2.9 Knockout

The VC125 strain that carries deletion (ok325) of the sequence between 3rd exon and 4th exon was ordered from the *C. elegans* Gene Knockout Consortium.

2.3 Computer Analysis

2.3.1 BLASTp searches

Biogenic amine receptors were identified in BLASTp searches using the Genbank BLAST server at http://ncbi.nlm.nih.gov/blast/ and the *C. elegans* and *C. briggsae* genomes BLAST server at http://www.wormbase.org/db/searches/blat. M03F4.3 was used as query sequence.

2.3.2 *Phylogenetic tree*

Dopamine receptor sequences were picked up as hits in BLASTp searches of the all organisms database. Their sequences were aligned with ClustalX v1.81, and a phylogenetic tree was constructed with NJPlot (using the neighbor joining method).

2.3.3 *Transmembrane Hidden Markov Model (TMHMM)*

Transmembrane prediction was conducted by using the TMHMM Server v2.0 at http://www.cbs.dtu.dk/services/TMHMM/ (Krogh *et al.* 2001).

3 RESULTS

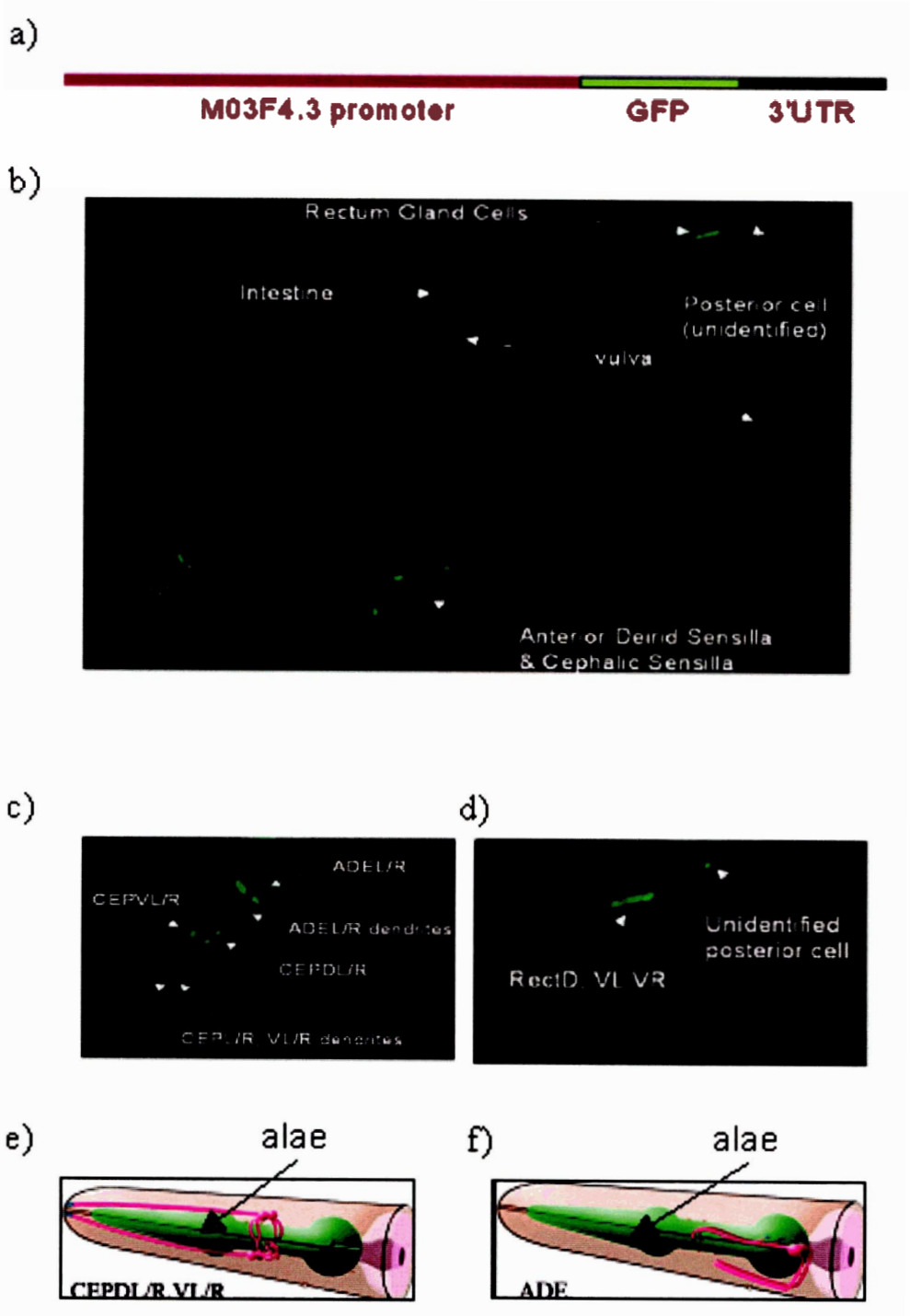
3.1 Expression of full length promoter *M03F4.3p(3700)::gfp*

A construct containing the GFP and full promoter region (~3700bp) upstream of M03F4.3 has been generated as shown in Figure 4a. This construct, *M03F4.3p(3700)::gfp*, was injected into the gonads of young adult hermaphrodites to create transgenic worms. The expression of ~3700bp promoter region, tagged with GFP, appeared in the anterior and the posterior regions, the intestine, and the vulva (Figure 4b). In the posterior region, the expression was observed in RectD, RectVL, RectVR, and one (unidentified) posterior cell (Figure 4d). In the anterior region, the expression was specifically observed in anterior deirid sensilla and cephalic sensilla (Figure 4c). The anterior deirid sensilla comprise two bilateral ADE neurons, which are located behind the second bulb of the pharynx, and the dendrites contain ciliated endings that travel into the deirid sensilla (White *et al.* 1986) (Figure 4e). Cephalic sensilla are made up of two dorsal and two ventral CEP neurons also located in the alae (Figure 4f). They contain a single long dendrite that extends from the cell body near the nerve ring through the length of the head where ciliated endings enter the cuticle near the nose of the animal (White et al. 1986, Perkins et al. 1986). The ADE and CEP neurons are known to be involved in dopamine expression/synthesis (Nass *et al.* 2003). In the nervous system of *C. elegans*, there are eight dopaminergic neurons – the four CEPs, the two ADEs and two PDEs (in posterior deirid sensilla) (Sulston *et al.*, 1975) and several genes that act in dopamine synthesis and signaling (Wintle and Van Tol, 2001), which is involved in locomotion and

egg laying (Schafer and Kenyon 1995; Weinshenker *et al.* 1995). The action of dopamine, which is transmitted through dopamine receptors on cell surfaces, plays a significant role in behavioral plasticity, or an alteration in behavioral state in response to changes in environmental stimuli (Sawin *et al.* 2000). In both invertebrate and vertebrate nervous systems, dopamine plays a critical role as a neuromodulator by altering intrinsic properties of neurons within circuits, both pre-synaptically (Harris-Warrick *et al.* 1995) and post-synaptically (Barnes *et al.* 1994). Dopamine has been implicated in locomotory and spatial learning behaviors in *Drosophila melanogaster* (Neckameyer *et al.* 1998) and in *Mus musculus* (Drago *et al.* 1998). Furthermore, the disruption of dopaminergic systems in human brains is linked to Parkinson's disease (Marsden *et al.* 1992) and schizophrenia (Hietala and Syvalahti *et al.* 1996). In mammals, five dopamine receptors have been identified, and they are characterized as G-protein-coupled, 7-TM eceptors (Vallone *et al.* 2000).

Figure 4a-f: Expression pattern of *M03F4.3p(3700)::gfp.*

(a) A *M03F4.3p(3700)::gfp* fusion construct was injected into hermaphrodites and transgenic animals were viewed using a Zeiss compound microscope with FITC filters for immunofluorescence. Expression was observed in **(b)** the intestine, vulva, anterior and cephalic deird sensilla, rectum gland cells and one of posterior cells. In the anterior region, expression was observed in **(c)** the ADEL/R neurons and dendrites. In the posterior region, expression was observed in **(d)** the CEPDL/R and CEPVL/R neurons and dendrites. The CEPDL/R and CEPVL/R together and ADEL/R are illustrated in **(d)**.


a)
M03F4.3 promoter
GFP
3'UTR
b)
Rectum Gland Cells
Intestine
Posterior cell (unidentified)
vulva
Anterior Deirid Sensilla & Cephalic Sensilla
c)
ADEL/R
CEPVL/R
ADEL/R dendrites
CEPDL/R
CEPDL/R VL/R dendrites
d)
Unidentified posterior cell
RectD, VL, VR
e)
alae
CEPDL/R VL/R
f)
alae
ADE

3.2 BLASTp searches for M03F4.3

The ~104 Mbp *C. briggsae* genome sequence has been used to find *C. briggsae* orthologs of *C. elegans*, to test for differences in expression pattern and function, and to identify conserved *cis*-regulatory elements in genes. A BLASTp of M03F4.3 has been performed against *C. elegans* and *C. briggsae* databases using the website http://www.wormbase.org/db/searches/blat. The search has revealed that *C. briggsae* CBG14571 is orthologous to M03F4.3, since 96% of their amino acid sequences are identical (Table 1a). *C. elegans* and *C. briggsae* diverged from a common ancestor roughly 100 million years ago (Coghlan and Wolfe 2002, Stein *et al.* 2003); nonetheless they are nearly indistinguishable morphologically (Nigon and Dougherty 1949) .

In addition, *C. elegans* genes such as K09G1.4 (*dop-2*), F15A8.5 (*dop-1*), C02D4.2 (*ser-2*), K02F2.6 (*ser-3*), Y22D7AR.13 (*ser-4*), C09B7.1 (*ser-7*) share high similarities based on amino acid sequence with M03F4.3 (Table 1b). The genes *dop-1* and *dop-2* code for dopamine receptors, whereas the genes *ser-2, ser-3, ser-4*, and *ser-7* code for serotonin receptors (Olde and McCombie 1997; Rex and Komuniecki 2002; Suo *et al.* 2002). In addition, the BLASTp of M03F4.3 has been performed against all organism database using the website http://ncbi.nih.gov/BLAST. Genes encoding *B. mori* (silkworm) tyramine receptor, *A. melifera* (honeybee) octopamine receptor, *O. mykiss* (rainbow trout) dopamine D2 receptor 1, and *C. familiaris* (dog) dopamine D2 receptor were revealed as closely related to M03F4.3 (Table 1c). This suggests that M03F4.3 gene codes for biogenic amine receptor, or more specifically, is a likely candidate for expression of a dopamine receptor. The amino acid sequences of *C. elegans* genes from the BLASTp search and the M03F4.3 were completely aligned using the CLUSTALX

program. The complete alignment of these sequences is illustrated in Figure 5, using the GENEDOC program, which reveals that M03F4.3, *ser-2*, *ser-4*, *ser-7*, *dop-1*, and *dop-2* share conserved 7-TM segments and the DRY, WPXF, WXXY, and NPXXY motifs, putting them all in the family A of rhodopsin/β2-adrenergic receptor-like receptors.

The expression patterns of the genes from the BLASTp results were compared with the expressional pattern of M03F4.3. Like M03F4.3, K09G1.4 is expressed in anterior deirid sensilla and cephalic sensilla (Suo *et al* 2003), which makes M03F4.3 a good candidate for expression of a dopamine receptor.

Table 1a-c: BLASTp best matches to M03F4.3.

(a) *C. briggsae* gene CBG14571 is the ortholog of M03F4.3, where 96% of the sequence is conserved. (b) *C. elegans* genes C02D4.2, F14D12.6, Y22D7AR.13, C09B7.1, F15A8.5, and K09G1.4 best match M03F4.3. (c) BmTAR, AmOAR, OmD2DR1, and CfD2DR are best matches of M03F4.3.

a)

C. briggsae COSMID NAME	GENE NAME	BLAST E-VALUE	% LENGTH (POSITIVES)
CBG14571	Unclassified	1.1e-291	96%

b)

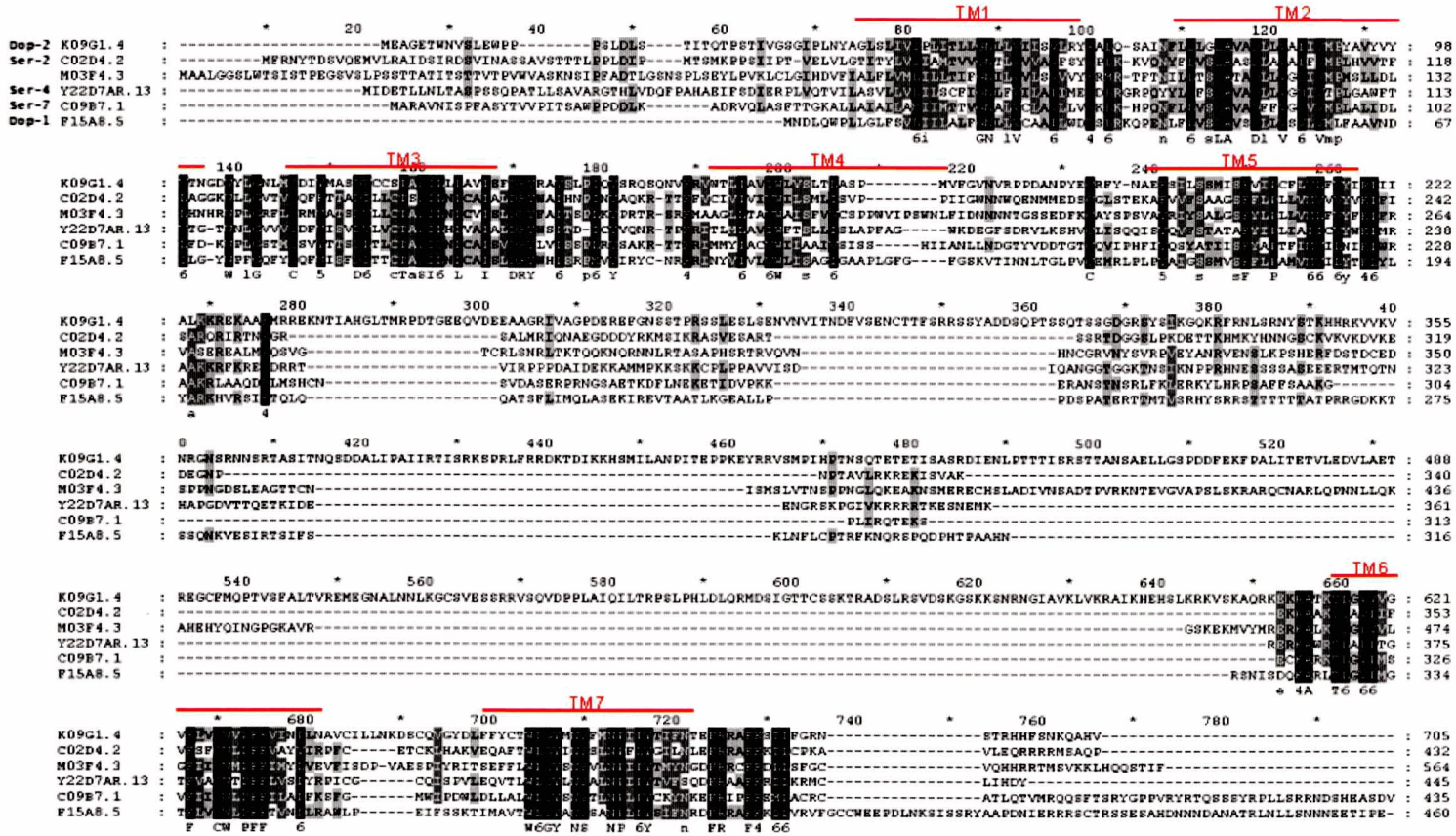
C. elegans COSMID NAME	GENE NAME/DESCRIPTION	BLAST E-VALUE	% LENGTH (POSITIVES)	LIGAND BINDING
C02D4.2	*ser-2*, tyramine receptor	4.2e-58	56%	Tyramine
F14D12.6	*ser-3*, serotonin receptor	2.0e-53	68%	Serotonin
Y22D7AR.13	*ser-4*, serotonin receptor	4.9e-46	50%	Serotonin
C09B7.1	*ser-7*, serotonin receptor	1.8e-54	53%	Serotonin
F15A8.5	*dop-1*, dopamine receptor	1.6e-44	66%	Dopamine
K09G1.4	*dop-2*, dopamine receptor	1.1e-43	49%	Dopamine

c)

SPECIES	GENE NAME/DESCRIPTION	BLAST E-VALUE	% LENGTH (POSITIVES)	LIGAND BINDING
B. mori	BmTAR, tyramine receptor	3.0e-56	50%	Tyramine
A. melifera	AmOAR, octopamine recepter	1.0e-55	49%	Octopamine
O. mykiss	OmD2DR1, D2-like dopamine receptor 1	9e-45	48%	Dopamine
C. familiaris	CfD2DR, D2-like dopamine receptor	7e-43	46%	Dopamine

Figure 5: Complete alignments of *C. elegans* genes and M03F4.3.

The sequences of *dop-1, dop-2, ser-2, ser-3, ser-4 and ser-7* are completely aligned with M03F4.3. All of them share contain similar sequences within the 7-TM segments, and conserved DRY, WXPF, WXXY, and NPXXY motifs.


TM1
TM2
TM3
TM4
TM5
TM6
TM7
Dop-2 K09G1.4
Ser-2 C02D4.2
M03F4.3
Ser-4 Y22D7AR.13
Ser-7 C09B7.1
Dop-1 F15A8.5

3.3 Expression of M03F4.3 regulatory elements

The expression of M03F4.3 in five regions must be governed by regulatory elements of the gene. Therefore, I investigated the regulatory elements for transcription of the gene in these regions. To achieve this, a series of deletions in the promoter region upstream of M03F4.3 were constructed and then injected into *C. elegans* hermaphrodites. These constructs are shown in Figure 6. The regulatory elements were determined by looking at expression changes in terms of different deletions. Table 2 illustrates the number of worms observed and the percentage of these worms that exhibited expression patterns. Following the injection of *M03F4.3p(2047)::gfp*, the expression of 2047bp promoter region appeared in the anterior deirid sensilla and cephalic sensilla, the unidentified posterior cell, the rectum gland cells and the intestine, but was lost in the vulva (Figure 7a-c).

The expression of 1447bp promoter region still appeared in the anterior deirid sensilla and cephalic sensilla, and the intestine, but was lost in the vulva and the posterior end (Figure 7d). The injection of *M03F4.3p(947)::gfp* resulted in the expression pattern similar to that of *M03F4.3p(1447)::gfp* (Figure7e). Furthermore, the expression of 447bp promoter region emerged in the anterior end and the intestine, and reappeared in one cell in the posterior end, whereas it remained lost in the vulva and the rectum gland cells (Figure 7f). However, the expression of 323bp was lost in the posterior cell again (Figure 7g). Finally, and interestingly enough, the expression of 200bp emerged in the intestine and the excretory gland, but disappeared in the anterior end, the rectum gland cells and the vulva; it also reappeared in the posterior cell (Figure 7g-i).

Figure 6: a series of promoter deletions and GFP construct fusions.

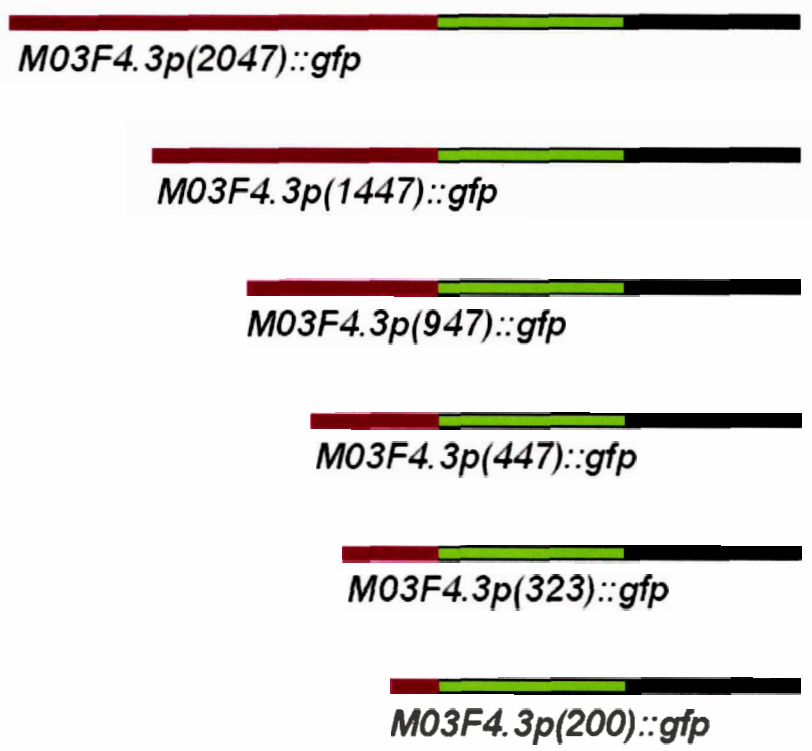

M03F4.3p(2047)::gfp
M03F4.3p(1447)::gfp
M03F4.3p(947)::gfp
M03F4.3p(447)::gfp
M03F4.3p(323)::gfp
M03F4.3p(200)::gfp

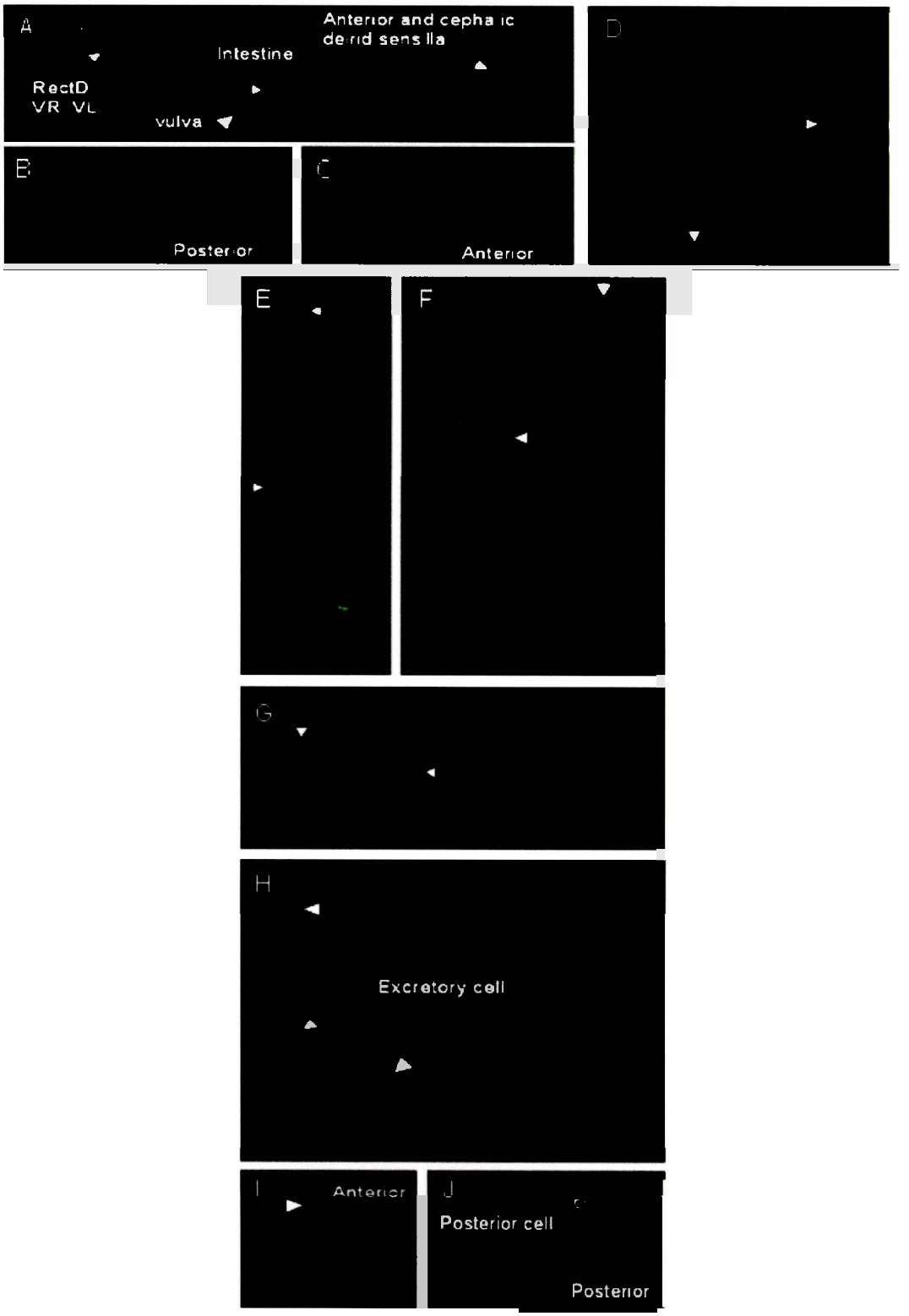
Table 2: Expression patterns of M03F4.3 with a series of promoter deletions.

30 transgenic worms in each line were observed for expression patterns.

CONSTRUCT	# WORMS	L3-L4 STAGE	ADULT STAGE	GFP EXPRESSION (% OBSERVED WORMS; STRONG (S), MODERATE (M), WEAK (W))						
				ADEL/R	CEPDL/R, VL/R	VULVA	INTESTINE	RectD, VR, VL	UNIDENTIFIED POSTERIOR CELL	OTHER
M03F4.3p(3700)::gfp	20	10/20	10/20	100%, S	100%, M	100%, S	100%, S	100%, S	100%, S	N/A
M03F4.3p(2047)::gfp	30	10/30	20/30	100%, S	100%, M	0	100%, S	67%, S	67%, S	N/A
M03F4.3p(1447)::gfp	30	10/30	20/30	100%, S	100%, M	0	100%, S	0	0	N/A
M03F4.3p(947)::gfp	30	10/30	20/30	100%, S	100%, M	0	100%, S	0	0	N/A
M03F4.3p(447)::gfp	30	10/30	20/30	100%, M	100%, M	0	100%, S	0	10%, S	N/A
M03F4.3p(323)::gfp	30	10/30	20/30	0	0	0	100%, M	0	0	N/A
M03F4.3p(200)::gfp	30	10/30	20/30	0	0	0	100%, M	0	45%, M	Excretory cell – 55%, S

Figure 7a-j: Expression patterns of the constructs.

The constructs as illustrated in Figure 6 were injected into hermaphrodites. Expression patterns of **(a-c)** *M03F4.3p(2047)::gfp,* **(d)** *M03F4.3p(1447)::gfp,* **(e)** *M03F4.3p(947)::gfp,* **(f)** *M03F4.3p(447)::gfp,* **(g)** *M03F4.3p(323)::gfp,* and **(h-j)** *M03F4.3p(200)::gfp* were viewed with Zeiss microscope. Arrows indicate expression, whereas arrowheads indicate the loss of expression.


A
Anterior and cepha ic
de rid sens lla
Intestine
RectD
VR VL
vulva
D
B
Posterior
C
Anterior
E
F
G
H
Excretory cell
I
Anterior
J
Posterior cell
Posterior

3.4 RNAi and knockout experiments

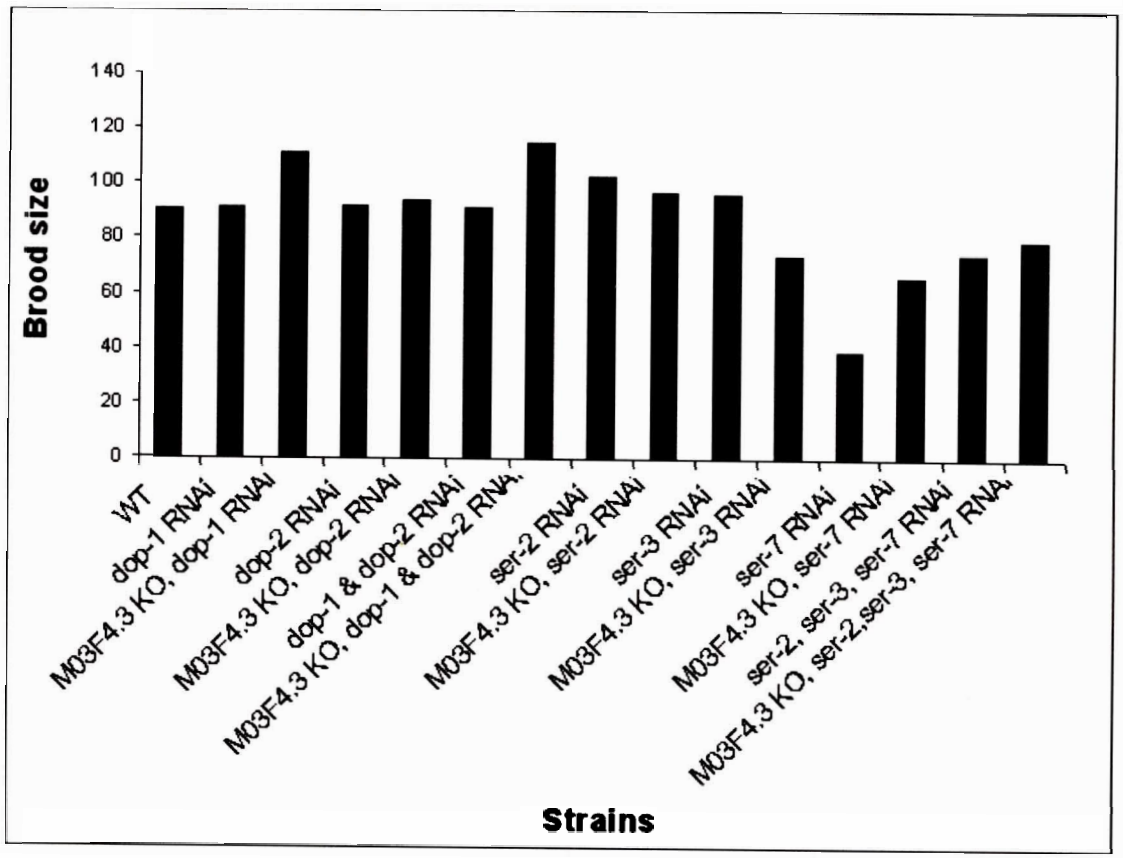
To investigate any impact on morphology, sexual and asexual reproductions, and locomotion due to the loss of M03F4.3, an experiment was conducted with RNAi against M03F4.3 in *C. elegans* hermaphrodites. Morphology and locomotion of the M03F4.3 RNAi worms were compared to those of the wild-type animals by generally observing their lengths and mobility. In addition, the M03F4.3 RNAi and wild-type worms were analysed for their ability to reproduce asexually, by directly examining the size of progeny derived from a single hermaphrodite. Hermaphrodites with brood size less than 10 are defective in asexual reproduction due to the loss of an essential gene. Sexual reproduction was also analysed, by observing the brood sizes of wild-type and M03F4.3 RNAi animals, and the number of males in their progeny. Progeny lacking males due to the loss of gene indicates that the gene is essential for sexual reproduction. The M03F4.3 RNAi animals, however, exhibited no phenotypic changes and were therefore superficially wild-type. Because RNAi experiments are effective on only 60% of genes, a knockout experiment was also conducted. The knockout strain, VC125, which contains the deletion of the sequence that spans from the 3rd exon and the 4th exon (*ok325*), was ordered from the *C. elegans* Gene Knockout Consortium. The strains were observed for phenotypic changes and were found superficially wild-type.

In addition, to determine whether the loss of combination of any *C. elegans* genes from Table 1b had any effect on wild-type or VC125 worms, RNAi against the genes were performed. The results again revealed superficially wild-type in asexual reproduction (as shown in Figure 8), sexual reproduction, locomotion, and morphology

(data not shown). The RNAi phenotypes of the *C. elegans* genes were not affected by the loss of M03F4.3.

Figure 8: RNAi data.

X-axis: the strains of wild-type or M03F4.3 KO animals with the loss of combination of any *C. elegans* genes (*dop-1, dop-2, ser-2, ser-3, ser-4, ser-7*). Y-axis: the brood size, or the size of progeny, derived from a single hermaphrodites. Blue bar indicates the number of progeny derived from a particular strain. The control of this experiment is the unfed wild-type animals, whose average brood size is ~95. The brood size derived from RNAi M03F4.3 hermaphrodite is also ~95 (data not shown).

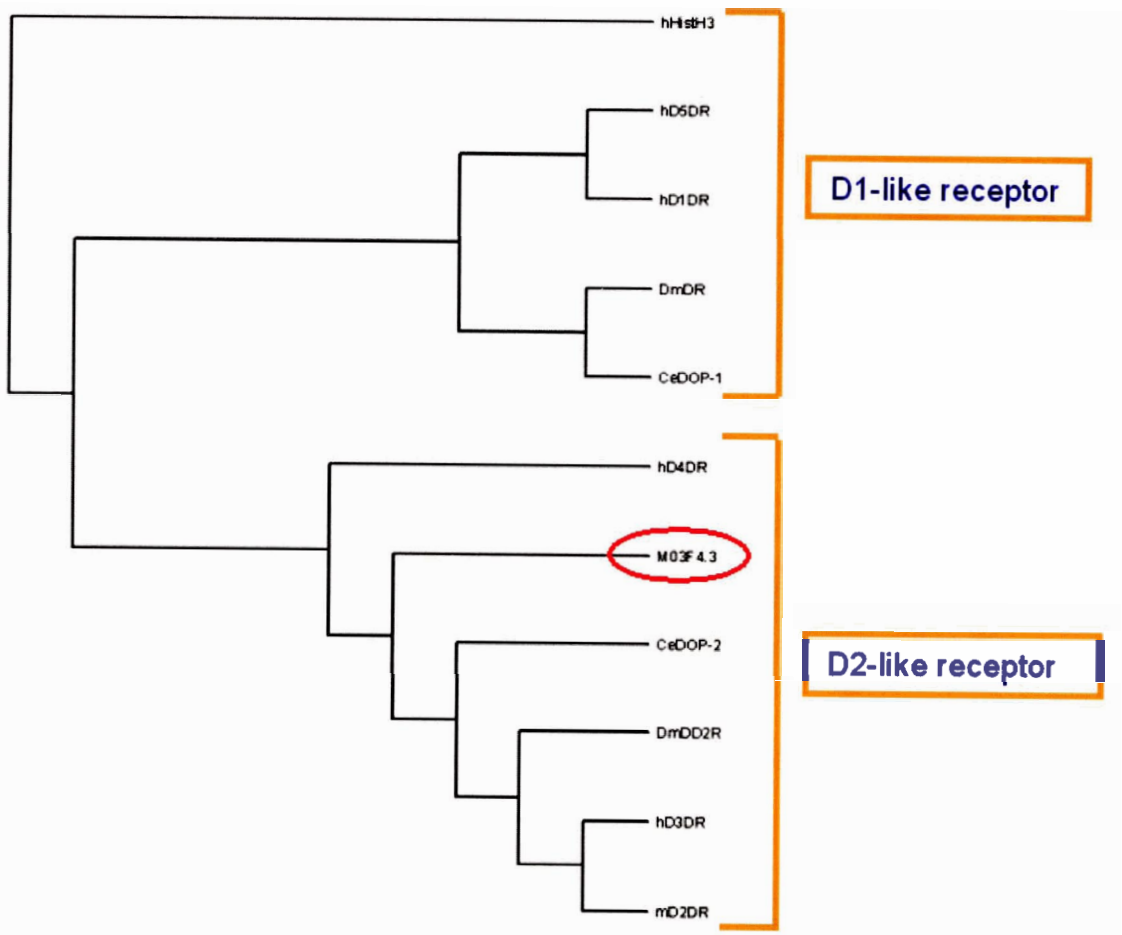

Brood size
140
120
100
80
60
40
20
0
WT
dop-1 RNAi
M03F4.3 KO, dop-1 RNAi
dop-2 RNAi
M03F4.3 KO, dop-2 RNAi
dop-1 & dop-2 RNAi
M03F4.3 KO, dop-1 & dop-2 RNA.
ser-2 RNAi
M03F4.3 KO, ser-2 RNAi
ser-3 RNAi
M03F4.3 KO, ser-3 RNAi
ser-7 RNAi
M03F4.3 KO, ser-7 RNAi
ser-2, ser-3, ser-7 RNAi
M03F4.3 KO, ser-2,ser-3, ser-7 RNA.
Strains

3.5 Classification of M03F4.3 as D1-like or D2-like dopamine receptor

The dopamine receptors are divided into two classes, D1-like and D2-like receptors. D1-like receptors are capable of activating adenylyl cyclase upon stimulation by agonists, which leads to an increase in intracellular cyclic AMP (cAMP), whereas D2-like receptors inhibit adenylyl cyclase. To investigate the class that M03F4.3 would likely fit in, M03F4.3 and the genes known to encode dopamine D1-like and D2-like receptors in *D. melanogaster, M. musculus*, and human were assembled into a phylogenetic tree (Figure 9). *C. elegans* DOP-1 is a D1-like receptor, whereas *C. elegans* DOP-2 is a D2-like receptor. As shown in Figure 9, M03F4.3 was clustered with D2-like receptors. This suggests that M03F4.3 is probably a D2-like dopamine receptor.

Figure 9: Phylogenetic tree of D1-like and D2-like receptors.

The C. elegans genome contains two dopamine receptors, F15A8.5/DOP-1 and K09G1.4/DOP-2, which are representative of the D1- and D2-like dopamine receptor subfamilies. D1- and D2-like dopamine receptor sequences were retrieved from Genbank and assembled into a phylogenetic tree with the distance-based neighbor-joining method using ClustalX. Human histamine receptor H3 was used as an outlier. "h" stands for human, "m" for mouse, "Dm" for *D. melanogaster*. GenBank Accession numbers are: DmDD2R = AAN15955.1, hD3DR = AAB08750.1, hD5DR = AAN01276.1, hD4DR = AAB59386.1, DmDR = AAF55030.2, hD1DR = AAB2673.1, CeDOP-1 = AAO91737.1, mD2DR = NP_034207.1, CeDOP-2 = NP_505478.2, hHistH3 = AAD38151.1


hHistH3
hD5DR
hD1DR
DmDR
CeDOP-1
hD4DR
M03F4.3
CeDOP-2
DmDD2R
hD3DR
mD2DR
D1-like receptor
D2-like receptor

4 DISCUSSION

4.1 M03F4.3 is a candidate dopamine receptor

In this study, I investigated the possible roles of M03F4.3 by first observing the expression of the construct with the putatively full length of its promoter fused to a GFP-encoding cassette. Expression was observed in vulva, intestine, rectum gland cells and one of posterior cells. The identity of the posterior cell remains unknown, since I did not investigate it in this study. The expression of the construct also appeared in anterior deirid and cephalic sensilla, which comprise ADE and CEP neurons, respectively, that play significant roles in dopaminergic signalling. These expressing cells could be laser-ablated to further confirm that they are ADE and CEP neurons. Since dopaminergic signalling plays a major role in normal sensory behaviors (i.e., foraging behavior and detection of bacterial lawn), a disruption in normal sensory function, due to lack of ADE or CEP neurons, should be detectable. For instance, animals lacking all four CEPs are defective in their basal slowing response (Sawin *et al.* 2000). After sensing and encountering bacteria, dopaminergic signalling inhibits locomotion, which is the basal slowing response. However, interestingly, the laser ablation of all four CEPs and two ADEs reveals no defect, whereas, the absence of all three classes of dopaminergic neurons (ADEs, CEPs, and PDEs) results in animals completely defective in the basal slowing response, which implies that these classes function redundantly to mediate the basal slowing response (*Sawin et al.* 2000). Furthermore, catecholamine-deficient (*cat*) genes (*cat-2* and *cat-4*), which have been identified to affect dopamine (Sulston *et al.*

1975), and a biogenic amine synthesis-defective gene (*bas-1*) lack normal sensory behaviors that are mediated by these dopaminergic neurons (Riddle *et al.* 1997, Loer and Kenyon 1993).

To confirm that M03F4.3 is a rhodopsin-like GPCR encoding gene, I performed a BLASTp search against the *C. elegans* and all organism databases. *C. elegans* biogenic amine receptors (*dop-2*, *dop-1*, *ser-2*, *ser-3*, *ser-4*, *ser-7*), *B. mori* tyramine receptor, *A. melifera* octopamine receptor, *O. mykiss* dopamine D2 receptor 1, and *C. familiaris* dopamine D2 receptor share high similarities, based on amino acid sequence, with M03F4.3. The *C. elegans* genes exhibit a structure that resembles 7-TM with DRY motif between TM segments 3 and 4, WXPF motif on TM segment 6, and WXXY and NPXXY motifs on TM segment 7. These conserved motifs are specific to all rhodopsin-like GPCRs. The biogenic amine receptor encoding genes in other organisms also reveal these conserved motifs (data not shown). The amino acid sequence of M03F4.3 is completely aligned with the conserved motifs in the *C. elegans* biogenic amine receptors, and in biogenic amine receptors in other organisms (data not shown), which confirms that M03F4.3 encodes a rhodopsin-like GPCR.

4.2 Expressed cells are controlled by different regulatory elements

To investigate the regulatory elements that govern expression of M03F4.3 in different cells, I created fusions of a series of deletions in the promoter upstream of M03F4.3 with a GFP encoding cassette. Following injection of *M03F4.3p(2047)::gfp*, the expression resulting from the 2047bp promoter region appeared strong in the anterior deirid sensilla, the cephalic sensilla, and the intestine, but was lost in the vulva in all 30 transgenic hermaphrodites (10 L3/L4 staged worms, 20 adults). Interestingly, nearly

67% of these transgenic worms showed strong expression in one of the posterior cell and in all rectum gland cells, while the other 33% completely lacked expression in the posterior end. The regulatory elements that control expression in vulva, posterior cell and rectum gland cells likely reside in the region between 3700bp at least 2047bp upstream of the start codon. The absence of expression in the posterior end in 33% of worms occurred based on two different possibilities. It likely depends on different regulatory elements within 2047bp upstream of the start codon, since the deletion in the promoter affected its expression. This implies that these worms lacking expression may be genetically different than the 67% worms with expression, which occurs after acquiring spontaneous mutations. The cells from these worms could be extracted for sequencing purposes and to investigate whether these cells reveal dissimilar sequences. The second possibility is that gene upregulation in the posterior cell and the rectum gland cells depends on the level of protein binding proteins such as transcription factors, the mechanism of which, however, is poorly understood.

Furthermore, the expression of *M03F4.3p(1447)::gfp* in all 30 transgenic worms observed still appeared in anterior deirid sensilla, the cephalic sensilla and the intestine. However, it remained absent in the vulva, and was completely lost in the posterior end. Therefore, the regulatory elements between 1447bp and 2047bp upstream of the start codon activate the transcription of the gene in the posterior cell and all rectum gland cells in 67% of transgenic worms. Like the expression pattern in *M03F4.3p(2047)::gfp,* certain levels of transcription factors may influence the upregulation of the gene in the posterior cell and the rectum gland cells. It is also likely that since the activation of

posterior cell depends on different segments of the promoter, it may require more than one type of transcription factors.

The expression of *M03F4.3p(447)::gfp* emerged in the anterior end and the intestine, and, interestingly, in 10% of transgenic worms, reappeared in the posterior cell, whereas it remained absent in the vulva and the rectum gland cells. This suggests that the regulatory element resides between 447bp and 947bp upstream of the start codon, which acts as suppressor of the gene activation in the posterior cell. The expression in 10% of the transgenic worms could be thought of as mosaic, which is an expression that occurs in one cell but not another. Mosaic is often caused by the loss of array (GFP) at an earlier cell division, and, therefore, some cells receive the array while others do not. It is, however, unlikely in this case since the expression was observed in all 30 transgenic worms from 4 different lines. The transgenic worms from 3 out of 4 different lines revealed expression in the posterior cell.

The expression of *M03F4.3p(323)::gfp* was lost in the posterior cell and the anterior end, indicating that the segment between 323bp and 447bp upstream of the start codon acts as activators of transcriptions in the posterior cell (in 10% of transgenic worms) and the anterior end. The expression of *M03F4.3p(200)::gfp* emerged in the intestine and unexpectedly, the excretory gland (in 55% of transgenic worms), but was remained absent in the anterior end and the vulva. Again, it reappeared in the posterior cell in 45% of transgenic worms. The suppressors of gene transcriptions in the posterior cell and the excretory gland (in 55% of transgenic worms) reside between 200bp and 323bp. Moreover, the expression in excretory gland is similar to the expression in *C.*

elegans dop-1. Lastly, the expression in intestine likely depends on regulatory elements that reside between the start codon and 200bp upstream of it.

4.3 M03F4.3 is a redundant gene

The function of M03F4.3 gene in *C. elegans* was investigated by performing the RNAi and KO experiments. Dopamine enables the worms to slow down in the presence of a food source in their environment, and to reproduce both asexually and sexually. The knockout of the gene, as well as the RNAi against the gene, showed no changes in reproduction behavior, locomotion, and morphology. It is likely that M03F4.3 is a redundant gene, since the lack of it showed no detectable effect in *C. elegans*. Phenotypic changes would be apparent if the gene were essential. The lack of dopamine receptor can lead to a defect in slow basal locomotion (worms would not slow down in the presence of food), high rate of egg-laying, defects in sexual reproduction and sterility in males.

Together these results show that M03F4.3 likely codes for dopamine receptor. The phylogenetic tree has also revealed the relationship between M03F4.3 and other D1-like and D2-like receptors in different organisms, therefore classifying it as a D2-like dopamine receptor. These results, however, are not sufficient to confirm the identity of the gene. Pharmacological characterization of the gene should be considered, since the testing application of different neurotransmitters (i.e., dopamine, serotonin, octopamine, tyramine) will aid in classification of the gene as dopamine receptor (see Future Directions).

5 FUTURE DIRECTIONS

5.1 Identify novel genes encoding transcription factors

In this study, I have identified the regulatory elements that control expression in the vulva, the anterior end, the posterior end, and the intestine. The transcription factors of these regulatory elements, and the mechanisms of DNA-protein interaction, however, have not been identified. Accurate transcriptional control is one of the fundamental steps in gene expression and regulation, and understanding the mechanisms behind transcriptional control is crucial in understanding of major cellular progresses (i.e. development, cell-cell communication) (Alberts *et al.* 1994).

A method called One-Hybrid System from CLONTECH Laboratories, Inc. should be considered as a first approach to identify novel DNA-binding proteins. The system provides basic tools for conducting an *in vivo* genetic one-hybrid assay, which is used for isolating novel genes encoding proteins that bind to a target, *cis*-acting regulatory element. The one-hybrid assay is based on the finding that several eukaryotic transcriptional activators are composed of functionally independent DNA-binding domains and activation domains, which allows researchers to construct gene fusions that, when expressed as fusion proteins in yeast, can simultaneously bind to a target sequence and activate transcription.

To understand the mechanisms behind transcriptional control in eukaryotes, it is necessary to investigate the cooperative nature of transcription factor interactions. The current view on transcriptional control in eukaryotes is the cell exerts this control through

cooperativity and synergy. Small combinations of signal-specific transcription factors bind to promoter DNA, which make possible the integration of several signalling pathways in the nucleus. The interactions between transcription factors on promoter DNA can be classified into three types – between DNA-binding factor and a non-DNA-binding factor, between DNA-factors adjacently located on the promoter, and between DNA-binding factors separately located on the promoter (Miller *et al.* 2003). The method ChIP-chip, which combines the techniques of chromatin immunoprecipitation and microarray hybridisation, can be used to elucidate the *in vivo* physical interactions of transcription factors with their chromosomal targets on the genome. This will provide a view of functional transcription factor-binding site interaction. The determination of transcription factors that control expression (i.e., of dopamine receptor) and mechanisms of DNA-protein interaction, can offer insight into misregulated expression that is common in many human diseases (i.e., Parkinson's disease, Alzheimer's disease) (Ly *et al.* 2000).

5.2 Pharmacological characterization of M03F4.3

Although M03F4.3 has been tentatively identified as dopamine receptor encoding gene, it has not been functionally characterized from any nematode. It is imperative to determine the pharmacological profile of M03F4.3, so ligands, such as dopamine, serotonin, tyramine, and octopamine, used to characterize other invertebrates, can be tested for their ability to displace [3H] LSD from membranes expressing M03F4.3 protein. This experiment measures the binding affinities of all biogenic amines to the protein of interest. A receptor should exhibit the greatest affinity to its corresponding ligand.

5.3 Promoter::GFP constructs – A tool for measuring effects of neurological drugs

Parkinson's disease is a neurodegenerative disorder that arises due to combinations of environmental and genetic factors, is characterized by resting tremor, bradykinesia (slowed movement), and rigidity. The regulated synthesis and release of dopamine by neurons contribute to normal motor function (Chase *et al.* 1998). The degeneration of dopaminergic neurons, and the subsequent loss of dopaminergic nerve terminals, cause the movement disturbances, which become more prominent with aging. Patients with Parkinson's disease are administered L-DOPA and other dopaminergic drugs for therapy, which is highly effective in managing early stages of the disorder. However, long-term treatment often goes along with a loss of drug efficacy and the occurrence of psychosis (Jenner 2003). Therefore, there are current studies to investigate non-dopaminergic agents, such as adenosin A2 antagonists (Richardson *et al.* 1997) as potential drugs in the therapy of motor disfunctions in Parkinson's disease. One might consider behavioural changes in worms that these drugs might induce. The use of promoter::GFP constructs can aid in detecting any expression alterations following the administration of these drugs. This can provide further insight into developing more improved therapy for patients with Parkinson's disease. Furthermore, a series of GFP fusions that would provide transcriptional indicators can be created in order to assess new drugs.

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WormBase web site, www.wormbase.org, releaseWS128, date 2 August 2004.

7 APPENDIX

A. 5' primers for M03F4.3 promoter

Amplification of 2047bp upstream of start codon [M03F4.3p(2047)]
5' GTTTGGAGGTTGCAAAATGTT 3'

Amplification of 1447bp upstream of start codon [M03F4.3p(1447)]
5' ACGTTTTTTAACCAGTCCAACA 3'

Amplification of 947bp upstream of start codon [M03F4.3p(947)]
5' TTTTTTTCCGTCAATATTGCT 3'

Amplification of 447bp upstream of start codon [M03F4.3p(447)]
5' AACAAAAAAAAGTTCTAAACT 3'

Amplification of 323bp upstream of start codon [M03F4.3p(323)]
5' TCTTGAATGGTCTGTTCGTTT 3'

Amplification of 200bp upstream of start codon [M03F4.3p(200)]
5' GCTCTGATCTTGTTTTTATT 3'

B. 3' primer for M03F4.3 promoter

5' TTCAACCCACCATAAGAAAATTG 3'