

TRANSCRIPTIONAL REGULATION OF AQP-8, A *CAENORHABDITIS ELEGANS*
AQUAPORIN EXCLUSIVELY EXPRESSED IN THE EXCRETORY SYSTEM, BY THE POU
HOMEBOX TRANSCRIPTION FACTOR CEH-6

Mah AK[§], Armstrong KR[‡], Chew DS[§], Chu JS[§], Tu DK[§], Johnsen RC[§], Chen NS[§], Chamberlin
HM[‡], Baillie DL[§]

[§]Department Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Drive,
Burnaby, British Columbia, Canada, V5A 1S6. [‡]Department of Molecular Genetics, Ohio State
University, 484 W. 12th Avenue, Columbus, OH 43210

Running Title: Transcriptional regulation of AQP-8 by CEH-6

Address correspondence to: Allan K. Mah, Department of Molecular Biology and Biochemistry, Simon
Fraser University, 8888 University Drive, Burnaby, British Columbia, Canada, V5A 1S6.

Tel. (604) 291-4597; Fax. (604) 291-5583; E-Mail amaha@sfu.ca

Due to the ever changing environmental conditions in soil, regulation of osmotic homeostasis in the soil-dwelling nematode *Caenorhabditis elegans* is critical. AQP-8 is a *C. elegans* aquaporin which is expressed in the excretory cell, a renal equivalent tissue, where the protein participates in maintaining water balance. To better understand the regulation of AQP-8, we undertook a promoter analysis to identify the *aqp-8* cis-regulatory elements. Using progressive 5' deletions of upstream sequence, we have mapped an essential regulatory region to roughly 300 bp upstream of *aqp-8*'s translational start site. Analysis of this region revealed a sequence corresponding to a known DNA functional element (octamer motif) which interacts with POU homeobox transcription factors. Phylogenetic footprinting showed that this site is perfectly conserved in four nematode species. The octamer site's function was further confirmed by deletion analyses, mutagenesis, functional studies, and electrophoretic mobility-shift assays. Of the three POU homeobox proteins encoded in the *C. elegans* genome, CEH-6 is the only member which is expressed in the excretory cell. We show that expression of AQP-8 is regulated by CEH-6 by performing RNAi experiments. CEH-6's mammalian ortholog, Brn1, is expressed both in the kidney and the central nervous system, and binds to the same octamer consensus binding site to drive gene expression. These parallels in transcriptional control between Brn1 and CEH-6 suggests that *C. elegans* may well be an appropriate model for determining gene

regulatory networks in the developing vertebrate kidney.

The *Caenorhabditis elegans* excretory system maintains osmotic homeostasis by expelling ionic and metabolic waste from the organism (1). The excretory system is comprised of four cells: the excretory duct cell, the binucleate excretory gland cell, the excretory pore cell, and the excretory (canal) cell. The excretory cell is a large fluid-filled cell consisting of a cell body which is located ventral to the isthmus worm pharynx. From the cell body, two long canals extend posteriorly and two shorter canals extend anteriorly; the four canals are joined at the cell body forming an H-shape. The surface areas of the four canals are greatly increased by a system of canaliculi which also provides exposure to the extracellular fluid-filled pseudocoelomic cavity (2). Developmental outgrowth of the excretory cell has been shown to be modulated by many of the same mechanisms that dictate neuronal guidance (3). Both of these tissues extend long cellular processes between their cell membranes and the epidermal basal lamina during development. Indeed, mutations in many genes, including *lin-17*, *unc-5*, *unc-6*, *unc-34*, *unc-53*, and *unc-73*, affect both neuronal and excretory cell development either by disrupting circumferential growth or by causing premature migrational termination of cellular processes (4). In addition, excretory cell tubulogenesis occurs *in vitro* using conditions intended for neuronal cell culturing (2).

Consistent with the excretory cell's role in osmoregulation, previous experiments have shown that the excretory duct cell pumping rates

in dauer larvae (a nematode diapause state induced by adverse environmental pressures) were inversely proportional to environmental osmotic pressures (5). Laser ablation of the excretory cell, duct cell, or pore cell leads to fluid retention within the worm (5). These two examples clearly demonstrate that the nematode excretory system is an osmoregulatory organ and therefore functionally analogous to the vertebrate kidney (1). The constrained developmental processes for such a unique structure and the physiological properties of the excretory cell make it an ideal tissue for studies on transcriptional regulation and how they relate to both renal and neuronal development. In addition, the excretory cell is the largest single cell in the worm making it ideal for studies of gross morphological defects.

In order to maintain fluid homeostasis between an organism and its environment and between tissues, members of the aquaporin (AQP)² family of water channels are expressed in various tissues and developmental stages in all forms of life to facilitate water movement across biological membranes. The existence of AQPs in virtually all cells allows for bidirectional passive flux of water across lipid bilayers, which in the absence of these proteins are essentially impermeable barriers. In addition, many cell types incorporate multiple different AQPs per cell and/or tissue. The spatial and temporal expression redundancy of AQPs may explain the relative lack of resultant gross phenotypes in AQP knockout studies. AQPs have been discovered in all types of organisms, from mammals to a recently discovered 270-aa homolog, AQPV1, in the *Chlorella* virus MT325s (6).

The first AQP cloned, the 28kDa protein AQP1, is a common protein in human red blood cell plasma membranes occurring at a level of approximately 120,000 – 160,000 copies per cell (7,8). Besides transporting water, many AQP members have the capacity to transport other small non-charged molecules. Selectivity of the channel for different uncharged solutes is derived from a proton filter region in the protein characterized by a pore-associated arginine residue in association with neighboring aromatic amino acids (9). Due to these different channel specificities, AQPs are grouped into two

functionally distinct classes: the aquaporins, which are exclusively water channels, and the aquaglyceroporins, which also have the ability to transport small non-ionic molecules such as glycerol and urea (10). AQPs contain a pair of signature domains, the NPA motifs (Asparagine-Proline-Alanine, or in some cases, Asparagine-Proline-Valine), which are essential for the pore structure and function (10). The locations of the NPA amino-acid residues allow passage of water through the pore. Due to hydrogen bonding interactions, the molecules travel through the pore in a single file manner (11). Overall, each AQP1 unit has the capacity to allow the passage of three billion molecules of water per second (11). An additional requirement for a functional aquaporin is a conserved fold in the protein which has been observed in both AQP1 and the bacterial aquaglyceroporin, GlpF (11). Some AQPs can be blocked by Hg²⁺ at a pore-associated cysteine residue (12).

In humans, seven out of the thirteen identified AQPs are expressed in various parts of the kidney in order to maintain osmotic balance and to prevent excessive fluid loss (13). The *C. elegans* genome contains eleven AQPs (*aqp-1* - *aqp-11*) (14). Like their mammalian counterparts, *C. elegans* AQP-4 (F40F9.9) and AQP-2 (C01G6.a), have been shown to be involved in fluid homeostasis. These results were obtained by physiological experiments determining changes in water flux as a result of the insertion of AQPs into *Xenopus laevis* oocyte membranes (15-17). In addition, AQP-4 was shown to be inhibited by Hg²⁺, much like other aquaporins (12,16,17). The expression patterns for the *C. elegans* AQPs 1 thru 8 have been determined previously (15). Three of the eight AQPs studied were demonstrated to be expressed in the excretory cell (*aqp-2*, *aqp-3*, and *aqp-8*) (15).

Prior studies have identified DNA regions containing functional *cis*-regulatory elements of various AQPs. Areas containing positive acting *cis*-regulatory elements for human AQP1 have been determined by analyzing the transcriptional activity of various length gene-upstream fragments (18). Regions containing negative transcriptional regulators of mouse AQP2 have also been determined (19). In addition, human

AQP4, which has two splice variants that lead to isoforms with distinct N-termini and pore permeability properties, has been shown to be under the control of alternate upstream regulatory sequences directly upstream of each splice variant (20). Studies in plants have also revealed regions containing *cis*-regulatory elements which modulate AQPs. An analysis of the AthH2 (PIP1b), an *Arabidopsis* AQP, upstream regulatory region revealed two phytohormone-induced enhancer-containing regions (21).

In this study we have determined a *cis*-regulatory element that is required for the expression of an aquaporin in *C. elegans*' excretory cell. Information pertaining to AQP expression and regulation in *C. elegans*' excretory system will provide complementary information to previous studies on transcriptional regulation of aquaporins and to also perhaps provide a basis for determining mechanisms controlling transcription in mammalian renal and neuronal tissues.

EXPERIMENTAL PROCEDURES

Nematode strains and maintenance - Strains were maintained at 20°C and all manipulations were conducted using standard methods (22). Strains used in this studies are shown in Table 1. We generated a stable Green Fluorescent Protein (GFP)-expressing line by subjecting (BC6835) *aqp-8-711::GFP-PEST* P₀ worms to 1,500R X-irradiation. Spontaneous transgene integrants were isolated by selecting for F₃ lines which produced 100% rescued *dpy-5* progeny thus producing (BC7032) *dpy-5(e907);Is1241 rCes[K02G10.7(-711)]::GFP-PEST +pCeh361*.

Transgene construction - DNA constructs were generated via fusion PCR as previously described (23). Promoter-containing sequences were fused upstream of the GFP coding region. The reverse promoter associated primer includes a segment complementary to the forward primer used for amplification of the GFP-reporter cassettes. GFP-coding cassettes used for expression pattern analysis are as follows: *pPD95.67 (GFP)*, *pPD95.75 (GFP)*, *pAF207 (GFP-PEST)*, and *pPD97.78 (Δpes-10::GFP)*. All GFP variants used are modified by the

addition of a 5'NLS, 3' *unc-54* UTR, S65C mutation, and additional synthetic introns. Site-directed mutagenesis of the motif site was carried out via nucleotide substitutions corresponding to residue changes in the motif in the forward primer. In all cases except the *Δpes-10::GFP* fusion construct and the AQP-8::GFP translational fusion construct, the reverse primer for upstream regulatory region amplification is aqp-8R:

agtcgacctgcagcatgcaagcttagaaacggatcgagaaaa.
The forward primers used for amplification of deletional constructs are shown in Supplementary table 1. The forward primers used for mutagenesis of the *cis*-regulatory element are as follows (mutated residues are underlined): aqp-8 oct G→A:
ttgccaaaattgacatactggaat and aqp-8 oct C→G:
ttgccaaaattggatactggaat. Primers used for tandem motif fusion to *Δpes-10::GFP* are as follows:
4XOCTR:
agtcgacctgcagcatgcaagcttatgcaaatatgcaaattta.
4XOCTL:

aattgcatataattgcatataattgcatataattgcatata). The reverse primer used for generating the translational AQP-8::GFP construct was AQP-8protB:

TTTCTACCGGTACCCTCAAGGGtccactactgtcactatactctgtca. The forward primer used for the translational construct corresponds to aqp-8-1.6kb (Supplementary table 1).

PCR constructs were co-injected with the *Dpy-5* rescuing construct, *pCeh361* (24), into the syncytial gonad of late L4 *dpy-5(e907)* worms. Individual wild-type F₁ worms were plated individually. Wild-type F₂ worms were selected to start the lines. In the case of generating multiple independent lines, each were analyzed separately and designated as individual segregants (Table 1).

Microscopy - A Zeiss Axioscope equipped with a QImaging camera and the appropriate optical filter sets were used for GFP expression pattern analysis. Worms were immobilized with 100mM sodium azide (in water) immediately prior to imaging. All images were taken at 400X with identical camera settings for all images(exposure times are indicated in the figures). Images were captured using QCapture

software and processed using Adobe Photoshop CS.

Sequence analysis - DNA and peptide sequence alignments were carried out using ClustalX (25) with default settings. The DNA sequence spanning the bases -283 to -234 upstream of the *C. elegans aqp-8* translational start site was used as a query in the Transcriptional Element Search System (TESS; <http://www.cbil.upenn.edu/tess>) to identify potential conserved transcription factor binding sites. Default parameters were used.

Electrophoretic Mobility Shift Assay (EMSA) - Nuclear and cytoplasmic extracts were isolated from N2 worms harvested in M9 buffer. The extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce). The synthetic biotinylated oligonucleotides used in this study include the consensus octamer oligonucleotide, 5'-ATTGCCAAAATTTGCATACTGGAAT - 3' and its complement 5' - ATTCCAGTATGCAAATTTGGCAAT - 3'. EMSA reactions were carried out using the LightShift Chemiluminescent EMSA Kit (Pierce). Samples were then loaded into an 8% non-denaturing polyacrylamide gel and electrophoresed in 0.5X Tris/Borate buffer at 100 V for 1 h. The entire gel electrophoresis apparatus was chilled using an ice-bath during operation.

RNAi - Adult BC6925 (AQP-8::GFP-expressing) worms were injected with 200 ng/ul of dsRNA corresponding to either *eri-1* (control) or a mixture of both *eri-1* and *ceh-6* dsRNA (experimental). The progeny of the injected worms were scored 48 hours post-injection for the presence of GFP fluorescence in the excretory cell using a standard image exposure time of one second with identical camera settings for all images.

Bioinformatic analysis - To identify genes that are potentially regulated by CEH-6 and the POU homeobox transcription factor DNA binding site (ATTTGCAT) in *C. elegans*, we carried out a bioinformatics search. We searched the putative upstream regions (in this analysis defined as

1,000 bp upstream of the translational start site (ATG)) of all *C. elegans* protein-coding genes, as well as the gene-upstream regions of genes in the related nematodes *C. briggsae* and *C. remanei*, for the motif ATTTGCAT. A *C. elegans* gene is considered if its *C. briggsae* and *C. remanei* ortholog both contain one or more octamer motifs as well. To achieve this, genome sequences of these three *Caenorhabditis* species and the predicated motifs were loaded into a MySQL database using the GFF3 format. Comparative analysis is done by Perl using Bio::DB::GFF module (26). Candidate *C. elegans* CEH-6-regulated genes were examined for their expression patterns by searching a *C. elegans* GFP expression database (Hunt-Newbury, et. al, submitted).

Statistical significance was determined by 10,000 random selections of the number of candidate genes with expression pattern and calculating the probability of the observed number of genes in the excretory cell. The probability was calculated by counting the number of times, out of 10,000 that a value is greater than or equal to the observed value over the total number of trials. Mathematically, this can be represented by letting v be the observed value and letting N be the set of excretory cell observations from random selections. S is the largest subset of N such that $\forall \sigma \in S, \sigma \geq v$. The resulting probability is $|S|/|N|$.

RESULTS

Identification of an excretory system expressed AQP - Gene transcriptional patterns were determined for each of the eleven *C. elegans aqp* members (*aqps 1-11*) by assaying expression patterns derived from *promoter::Green Fluorescent Protein (GFP)* fusions *in vivo* (data not shown). Consistent with previous evidence (15), *aqp-8* is the only AQP that had its expression localized exclusively to the excretory system of the worm (Fig. 1). The intergenic region between *aqp-8* and its closest upstream gene-neighbor, *K02G10.1*, is 2,220 bp. Our initial *aqp-8* upstream region was defined as the 1,556 bp region from -1575 to -20bp immediately upstream of the *K02G10.7* translational start site (Fig. 1A). Expression of *aqp-8* also appears to be localized to an

additional cell. We presume, by the location of the additional cell, that this cell may be the excretory gland cell. Since many genes are transiently expressed for specific physiological functions and processes, we assayed for the temporal activity of the *aqp-8*'s upstream regulatory region using the *GFP-PEST* reporter (*pAF207*, kindly provided by A. Frand) (27) (Fig. 1B). The PEST sequence, (Pro-Glu-Ser-Thr) is a signal for rapid degradation of proteins, which was discovered as a conserved sequence from multiple alignments of known short-lived proteins (28). The coding sequence for the PEST sequence was inserted directly upstream of the 3'UTR towards the C-terminal portion of *GFP*, producing a *GFP* variant with an *in vivo* half-life of less than one hour (27). The *aqp-8p::GFP-PEST* expressing worms displayed an identical spatial pattern to the worms carrying the usual *aqp-8::GFP* construct, but due to the short half-life of the *GFP-PEST* construct, we were able to determine that *aqp-8* is transcribed only in the interval between the first larval stage and early adulthood. The relative levels of expression in the excretory cell and the excretory gland cell appeared to be similar to each other (Figs. 1A,B). Expression patterns derived from extrachromosomal arrays may be confounded by somatic loss of the transgene (leading to mosaicly expressing transgenes). Therefore we confirmed the expression pattern of *aqp-8* by generating a genome-integrated *aqp-8p::GFP* transgenic line to prevent the sporadic loss of the transgene in somatic tissue (Fig.1C). These expression data are consistent with the *C. elegans* developmental Serial Analysis of Gene Expression (SAGE) profiles of *aqp-8* (29) which show *aqp-8* mRNA production starting in L1 larval stage and intensifies until L4 when the level of transcription tapers off. SAGE data, corresponding to various libraries derived from Fluorescence Assisted Cell Sorting (FACS) derived *C. elegans* embryonic cells, revealed that *aqp-8* is expressed at a low but detectable level in each of the purified oocyte, embryonic, and sorted AFD neuron libraries (29). The *aqp-8* gene locus, *K02G10.7*, encodes two splice variants which differ by an alternatively spliced fourth exon. The smaller isoform, *K02G10.7a*, encodes a 258 amino acid protein whereas *K02G10.7b* encodes a slightly larger

294 amino acid protein. Both AQP-8 isoforms have been confirmed by ESTs (Fig. 2A) (30). *K02G10.7a*, contains five possible membrane spanning regions whereas the larger b variant contains six membrane spanning regions.

The closest mammalian homolog of *C. elegans* AQP-8 is AQP10, an aquaglyceroporin predominantly expressed in the jejunum glands (Crypts of Lieberkühn), and the duodenum epithelia. The jejunum glands function to secrete various digestive enzymes and also contain mitotically active stem cells for the purpose of epithelial regeneration. A possible role for AQP10 is to regulate osmolarity in these regions of the small intestine which are known to be subject to considerable changes in solute concentrations. In particular, stringent regulation of duodenum osmolytes may be due to large changes in solute concentrations as undiluted stomach contents are passed directly through to it (31). Like AQP-8, vertebrate AQP10 has two isoforms with five (30kDa) and six (35kDa) transmembrane domains respectively (32). Though *bona fide* transcripts have been identified for the shorter splice variants of both AQP-8 and AQP10, it is possible that these isoforms are non-functional. Unlike AQP-8, AQP10 can conduct both water and glycerol (15,32). It has been suggested however that AQP-8 may be important for adaptation to osmotic stress as its expression levels have been observed to be induced significantly when worms are placed under hypotonic stress (15). The two NPA domains in *K02G10.7b* are located between the transmembrane segments II/III and V/VI which correspond to the locations of the NPA domains in human AQP1. A null mutant of *aqp-8* (*tm1919*) does not show any obvious structural defects in the excretory cell or any assayable response to changes in osmotic pressures (data not shown). Likewise, treatment of nematodes with RNAi corresponding to *aqp-8*, clone *sij_K02G10.7* (33), did not result in any obvious change in response from wild-type worms when placed in media containing different levels of salinity (data not shown). Though loss of AQP-8 itself does not lead to an observable phenotype, quadruple mutants of *aqp-2*, *aqp-3*, *aqp-4*, and *aqp-8* have been shown to lead to worms with impaired mobility when subjected

to hypotonic environments (15). To determine whether AQP-8 remains in the excretory cell after translation, we generated a K02G10.7 translational GFP fusion. This construct consisted of *aqp-8*'s 1.6kb upstream region and coding sequence with GFP (from the plasmid *pPD95.75*) fused at the C-terminus of AQP-8. Localization of AQP-8 was identical to the expression pattern revealed by the transcriptional GFP fusions albeit displaying a lesser level of fluorescence than worms carrying the *promoter::GFP* (and *promoter::GFP-PEST*) constructs (Figs. 1D,E). The lower fluorescence level of the translational reporter construct relative to the transcriptional reporter constructs may be attributable to a higher protein turnover of the AQP-8::GFP protein relative to untagged GFP.

Determination of upstream regions required for excretory cell expression of aqp-8 - The initial expression pattern analysis of *aqp-8* was examined for two constructs *in vivo* using the 1.6kb fragment fused to both GFP and GFP-PEST. To map the upstream DNA elements responsible for the excretory system specific expression of *aqp-8* in *C. elegans*, a series of fragments consisting of progressive 5' deletions of *aqp-8*'s upstream regulatory region were fused to the GFP or GFP-PEST coding cassettes. A *cis*-regulatory element was initially localized to a region spanning the nucleotides -342 → -207. A further round of deletions within the defined window resolved the *cis*-regulatory element containing region to an interval between -279 → -261. GFP expression levels and patterns of the transgenics were consistent with the original 1.6kb *aqp-8* upstream constructs' expression pattern until loss of expression in -261 constructs and all subsequent shorter constructs (Fig. 2). From the deletion analysis of the upstream regulatory region, we have determined that *aqp-8* expression is modulated by at least one *cis*-regulatory element located within the 19bp interval spanning -279 to -261bp relative to the translational start site of *aqp-8*.

Phylogenetic footprinting of the the aqp-8 gene-upstream region - Although the morphologies of the two nematode species, *C. briggsae* and *C. elegans*, are similar, analysis of

the mutation rates in gene ortholog pairs have revealed that the two species have diverged approximately 80-110 million years ago (34). Since their general body plan and developmental programs have changed very little over the long evolutionary distance, most DNA coding regions and functional non-coding DNA elements are likely to be under purifying selection. With the availability of both of their genomic sequences, we can compare orthologous non-coding genomic regions to identify conserved functional nucleotide regions. In addition to the extensively curated *C. elegans* (14) and annotated *C. briggsae* (34) genome sequences, the recent availability of two other closely related nematode genome sequences, *C. remanei* (http://genome.wustl.edu/pub/organism/Invertebrates/Caenorhabditis_remanei/) and *C. brenneri* (http://genome.wustl.edu/pub/organism/Invertebrates/Caenorhabditis_PB2801/), allows for a multiple alignment of the four species' orthologous upstream regions. A ClustalX alignment revealed a perfectly conserved 10bp region between the four nematode species (AATTTGCATA) which falls within the region (-279bp to -261bp), in *C. elegans*, determined by the upstream regulatory region-deletional analysis (Fig. 3). The distances between the start of the motif and the translational start sites were fairly well conserved with the positions in *C. remanei* (-277bp) and *C. briggsae* (-283bp). The similarity in the upstream distance of the motifs indicates that the position of the element relative to the translational start site may be important for the element's ability to modulate gene expression. Positional preference of *cis*-regulatory elements upstream of the gene translational start sites in *C. elegans* have been observed with the X-box (35), E-box, SMAD, and CdxA (36) transcription factor binding motifs.

Determination of conjugate transcription factors - To determine whether there were any previously defined DNA motifs from other organisms located within the 19bp window defined by the transgenic constructs, the sequence spanning the bases -283 to -234 was used as a query in Transcriptional Element Search System (TESS; <http://www.cbil.upenn.edu/tess>). The search

results revealed an eight-nucleotide non-palindromic sequence located at the bases spanning -268 to -261 relative to the translational start site of *aqp-8*, again falling within the window defined earlier. This site corresponded to a POU (*pit*, *oct*, *unc*) transcription factor consensus DNA binding site commonly referred to as the octamer motif (ATTTGCAT). The POU homeobox transcription factors have a carboxy-terminal bipartite DNA-binding region which consists of a POU-specific (POUs) domain, a flexible linker region, and a downstream homeobox (POUh) domain (37). The POU region associates with the 5'-half of the DNA motif, while the POUh, associates with the 3'-half (38). Several POU transcription factors have been shown to regulate important developmental processes in the vertebrate embryo. The entire *C. elegans* genome encodes only three POU transcription factors, *unc-86*, *ceh-6*, and *ceh-18* (14). Expression pattern analyses and functional characterizations of each *C. elegans* POU transcription factor member have been carried out in previous studies. One POU transcription factor, UNC-86, is a nuclear protein which plays a role in neuronal development (39). UNC-86 has been shown to be expressed predominantly in mechanosensory, odorsensory, and chemosensory neurons where it controls neuroblast specification (40). The consensus UNC-86 binding site sequence in *C. elegans* is CATnnnT/AAAT which is identical to the binding site of its mammalian ortholog Brn3 (41). Another POU transcription factor, CEH-18, is required for directing proper gonadal sheath development and function. Loss of CEH-18 leads to defective oocyte maturation (42,43). Among the three *C. elegans* POU transcription factors, CEH-6, a class III POU, is the only one that is expressed in the excretory cell. CEH-6 has been shown to be expressed early in the development of the excretory cell in addition to four pairs of head neurons, the SABV motorneuron cell pair (SABVL and SABVR) and several cells in the vulva and tail (44). The expression of *ceh-6* not only overlaps the expression of *aqp-8*, but also precedes the expression of *aqp-8*, thus fulfilling the spatial and temporal expression pattern criteria as a modulator of *aqp-8* expression. Null mutants of

ceh-6 and post-transcriptional disruption by RNAi of *ceh-6* lead to phenotypes which resemble mutants with impaired development of the excretory cell (33,45). Two additional *aqp-8::GFP* fusion constructs were generated with the 5' end of the construct terminating at -272 (containing the whole octamer site within the construct) and another with the 5' end of the construct terminating at -267 (excluding the terminal nucleotide of the octamer site within the construct) to confirm the function of the site. While the -272 construct drove expression of the GFP in the excretory cell, the -267 construct failed to drive expression of the GFP-coding cassette (Fig. 4A). This gives strong evidence that the site is required for recruiting *trans*-acting factors to mediate gene expression in the excretory cell. To further characterize this site, we constructed a *promoter::GFP* fusion construct containing mutated octamer sites. The first mutagenized construct consisted of a G→A change at position -264 (Fig. 4A,B). The single residue change did not lead to a change in the GFP expression level. Previous studies have shown that the site, ATTTACAT and/or its reverse complement, ATGTAAAT, are functional POU transcription factor binding sites (46,47). Changing the adjacent downstream residue in the octamer site (ATTTGCAT → ATTTGGAT) lead to a complete loss of GFP expression (Fig. 4A,B). Crystallographic studies of the Oct1 POU domain bound to an octamer motif containing DNA strand have shown that the -263 G→C change affects a DNA binding site amino acid in the POU region of the transcription factor (38). The POU homeobox DNA interacting amino acid residues which contact this region of the DNA octamer motif are highly conserved among POU transcription factor homologs in both mammals and *C. elegans* (data not shown).

Expression specificity of the POU TF binding site - Because POU binding sites have been shown to mediate expression in a variety of vertebrate cells, we tested the ability of the octamer motif to drive expression of the GFP reporter independently of other *cis*-linked downstream factors associated with *aqp-8*. To this end, we used the $\Delta pes-10::GFP$ cassette (*pPD97.78*, kindly provided by Fire, A. Z.,

Stanford University School of Medicine). The $\Delta pes-10::GFP$ cassette is composed of the minimal promoter from *C. elegans pes-10* fused to a GFP reporter. Alone, the $\Delta pes-10::GFP$ reporter construct has minimal transcriptional activity. The minimal promoter can be activated by the presence of upstream enhancers for the purpose of determining the transcriptional activities of the introduced *cis*-linked elements. Using the $\Delta pes-10::GFP$ construct, we tested for the ability of the putative *cis*-regulatory element to act as an excretory system enhancer. We fused four tandem repeats of the 10bp nematode conserved sequence (AATTTGCATA) to the 5' end of the $\Delta pes-10::GFP$ cassette (Fig. 4C). The resulting GFP fluorescence, driven by the tandem repeats fused to the basal promoter, was observed in the excretory cell beginning at L1 and continuing into adulthood much like the expression pattern of the *aqp-8::GFP* constructs, albeit at a much lower level than the initial GFP-expressing constructs (Fig. 4C). We did not detect expression in the additional cell identified earlier as possibly the excretory gland cell. This may be due to expression of GFP in the additional cell being below the detection level of the microscope configuration used or that expression in the gland cell is controlled by a separate *cis*-regulatory element. Additional GFP fluorescence arising from this construct was detected in two anterior neurons. The expression in the anterior neurons indicates that the octamer motif may also be responsible for recruiting transcription factor(s) responsible for driving expression in those neurons. The lower excretory cell expression level can be explained by the possibility of additional expression enhancing *cis*-regulatory elements which exist downstream of the octamer element that were not included in the sequence fused to the $\Delta pes-10::GFP$ cassette. Another possible explanation is that the expression level may be dependent upon the distance between the *cis*-regulatory element and the translational start site.

Binding of a nuclear protein to motif fragment
 - Previous studies have shown that the Brn3, a POU homeobox transcription factor, complexes with its cognate DNA recognition motif stably even in non-physiological conditions (48). This property allows for the complex to be resolvable

by *in vitro* binding reactions. To investigate whether the putative POU site is able to bind *C. elegans* nuclear proteins, an Electrophoretic Mobility Shift Assay (EMSA) was conducted using the complementary biotinylated 25-bp oligonucleotides containing the octamer site along with flanking sequence as probes. The probes, when incubated with *C. elegans* mixed stage nuclear protein extract, led to band shifts when run on a non-denaturing acrylamide gel. To determine if the binding was specific, unlabeled competitor oligonucleotides with the identical sequences were coincubated with the biotinylated probes in separate binding reactions. The presence of a 1000-fold excess of identical unlabeled probe led to a decrease in the amount of protein bound to the biotinylated probe (Fig. 6A,B). Band shifts were also observed in reactions using cytosolic extracts. Though the shifted band appeared to be the same size, implying that the bound protein is most likely the same, the amount of DNA recruited in the cytosolic fraction appeared to be significantly more than in reactions with nuclear extract. The presence of 1000-fold excess probe in the cytosolic fraction also leads to a decrease in the amount of protein bound by the biotinylated probe (Fig. 6A,B). We also performed an EMSA reaction containing nuclear protein extract in the presence of the same octamer-containing biotinylated oligos in addition to a 250-fold excess of an unrelated dsDNA probe (5' TTTTGTCCCTCGTGGGAGACACAT annealed to its complementary sequence, 3' 3' ATGTGTCTCCCACGAGGGACAAAA 5'). The presence of the excess unrelated dsDNA did not affect the octamer-site probe / nuclear protein binding interaction (data not shown). These results suggest that the element and some flanking sequence are sufficient to recruit a *trans*-acting factor which is present in both the cytoplasm and the nucleus to the DNA sequence.

Confirmation of CEH-6 / octamer interaction
 - To verify the dependence of *aqp-8* transcription on CEH-6, we knocked down CEH-6 in an AQP-8::GFP-expressing background using RNAi (49). We performed a double RNAi experiment using both *aqp-8* and *eri-1* dsRNA.

eri-1 encodes an siRNase which expresses in *C. elegans* gonadal and nervous tissue. Knocking down ERI-1 leads to a pronounced RNAi effect in the tissues which ERI-1 is expressed (50). Treatment of the AQP-8::GFP-expressing worms with *eri-1* dsRNA (n=30) failed to down-regulate AQP-8::GFP expression in any worms, however, the double RNAi treatment of AQP-8::GFP-expressing worms with both *eri-1* and *ceh-6* dsRNA (n=30) led to a consistent complete elimination of GFP expression in the excretory cell when scored 48 hours post-injection (Fig.5). The double dsRNA treatment led to developmental arrest at the L2 stage larva as a result of knocking down *ceh-6* expression. This phenotype is consistent with the phenotype of the *ceh-6(mg60)* null mutant showing that the double dsRNA treatment is effectively knocking down *ceh-6* expression. Developmental arrest was not observed for worms injected with *eri-1* dsRNA alone (scored at 72 hours post treatment, data not shown). Taken together, we show that CEH-6 is the POU transcription factor that regulates *aqp-8* via binding to its cognate octameric POU homeobox transcription factor binding site.

Determination of other candidate genes modulated by ceh-6 – With the confirmed interaction of CEH-6 with the octamer element, we searched for instances in which the octamer motif was conserved between these three nematode species: *C. elegans*, *C. briggsae*, and *C. remanei* to determine other potentially co-regulated genes. Four sets of analyses were done according to different filtering criteria. The common criteria among all four sets were that the gene is orthologous in *C. elegans*, *C. briggsae*, and *C. remanei*; and that there is at least one octamer motif predicted in the upstream regulatory region. The other criteria specific for each set are summarized in (Table 2). 107 genes were identified with perfect motif matches among the three genomes under the most relaxed condition and 44 genes under the strictest condition (Supplementary Table 2). Of the candidate genes identified, *promoter::GFP* expression pattern data has been generated for 19 (relaxed condition; all) and ten (strictest condition; SE) of the upstream regulatory regions using *promoter::GFP* reporter

constructs respectively (29) (Table 2). Three genes that contain excretory cell expression are consistently observed for all gene sets (Table 3). In order to determine whether octamer motifs are enriched in genes expressed in excretory cells, we carried out statistical analysis calculating the significance of observing three excretory cell expressions. We found that the probabilities were 0.3556 and 0.0857 for the most relaxed and most stringent conditions respectively (Table 2). Of the 28 gene candidates identified with the least stringent conditions (SE) (Table 3), 17 genes have associated GO terms (Supplementary Table 3) and thus provides a starting point for determining the physiological role of AQP-8 and its orthologs.

DISCUSSION

Previous work has shown that approximately 12% of *C. elegans* excretory expressing genes may be transcriptionally regulated by the EX-1 *cis*-regulatory motif in conjunction with its cognate transcription factor, DCP-66 (51). DCP-66 is a widely expressed transcription factor and has been shown to be expressed in neurons, the pharynx, body wall muscle, excretory cell, and vulva (51). Close homologs of DCP-66 generally act as transcriptional repressors, though in the case presented by Zhao *et al.* 2005, DCP-66 clearly acted as a positive regulator of gene expression. In addition, the report by Zhao *et al.* 2005 discover two other *cis*-regulatory elements that mediate gene expression in the excretory cell.

Although the expression of AQP-8 is tightly regulated in the excretory system, all of the transcriptional regulatory elements that contribute to its expression pattern have not been elucidated. Previous studies have reported the expression pattern of AQP-8, but we have discovered that expression is found in another cell which we presume to be the excretory gland cell. Moreover, using a reporter protein with a limited half-life, we have determined a precise temporal window of expression for *aqp-8*. Here we present a novel model of transcriptional regulation which is the interaction of the POU homeobox transcription factor, CEH-6, with an

octamer motif in *aqp-8*'s upstream regulatory region.

The binucleate excretory gland cell is the product of a cell fusion. Although the gland cell has been proposed to function as a secretory organ, based on its morphology, it is the only non-vital cell in the excretory system. Laser ablation of the gland cell does not lead to any obvious shortcomings in worm development or function under standard laboratory conditions (5). Clues as to the excretory gland cell's role in the worm can be inferred by virtue of its possible co-developmental regulation with the excretory cell.

Expression pattern analysis using GFP based reporters generally agreed with SAGE profiles derived from either staged worms of FACS-based isolation of certain cell types. Surprisingly, *aqp-8* message was also detected in AFD-specific libraries (29). Analyses of mutants and cell-ablations of the AFDs, have determined that the ciliated neurons AFDL and AFDR are required for thermosensation in *C. elegans* (52,53). The developmental lineages of the excretory cell and the AFD neurons diverge at the four-cell stage, a stage that has not been used for deriving the AFD SAGE library (54). A possible reason for the SAGE tag arising in the AFD-specific library is that the tag might represent message derived from background foreign cell contamination during the FACS stage.

By assaying for the expression activity of progressive deletions of the *aqp-8* upstream regulatory region, we have delimited a region important for the expression of *aqp-8*. We used sequenced closely-related rhabditid genomes to facilitate interspecies comparisons of *aqp-8*'s upstream regulatory region. The alignments, in conjunction with the window derived from the promoter truncation analysis, allowed us to identify a single *cis*-regulatory element required for expression of AQP-8. The *cis*-regulatory element corresponds to the octamer motif, a DNA sequence known to recruit POU homeobox transcription factors for activation of downstream genes. We demonstrate that the transcription factor responsible for *aqp-8* excretory cell expression is CEH-6 using a double RNAi strategy which enhances the RNAi effects in certain tissue-types. We find that

CEH-6 is found in both the cytosolic and nuclear protein extracts. The presence of a cytosolic binding partner for the octamer site is not surprising due to the fact that POU homeobox transcription factors have highly conserved basic nuclear localization (NLS) and leucine-rich hydrophobic nuclear export signals (NES) (55,56). An alignment of the human POU homeobox TFs, Oct6 and Brn1, against all three *C. elegans* members reveals that the two localization signal sequences are conserved in the nematode POU proteins (data not shown). To verify the cytosolic localization of CEH-6, we assayed for the expression pattern of a CEH-6 translational GFP fusion construct. The construct revealed that the protein is intracellularly localized both to the nucleus and the cytoplasm of the excretory cell (data not shown). The nuclear export signal found in POU TFs has been demonstrated to act in a CRM1/Exp1-dependent manner. The *C. elegans* genome contains an ortholog of CRM1/Exp1, IMB-4 (importin-beta-like protein-4, ZK742.1). The GFP signal resulting from the transgene *ZK742.1::GFP* was too weak to be detected and therefore we could not determine if CEH-6 and IMB-4 are cellularly co-localized. The EMSA results also indicate a greater abundance of the DNA-interacting protein in the cytoplasm than in the nucleus. This may indicate a nuclear export rate for CEH-6 that exceeds its nuclear import rate. The existence of these localization signals in CEH-6 likely facilitates rapid transient transcriptional modulation of target genes via nucleo-cytoplasmic shuttling with the cytosol acting as a repository.

Analysis of the expression pattern derived from the tandem POU motif repeat fused to the *Apes-10::GFP* reporter revealed that the motif also drove expression in the AUA (AUAL and AUAR) and AVH (AVHL and AVHR) neuron pairs (Fig. 4D). The AUA neurons are involved with integrating environmental cues to dictate social versus solitary feeding choices (57). Though AQP-8 was not detected in the AUA neurons, AQP-8 may play a role in integrating osmoregulatory cues in conjunction with these cells. The function of the AVH neurons is unknown, but it appears that both the AUA-type and AVH-type neurons co-express the glutamate receptor GLR-4, and also a splice variant of the

tyramine receptor SER-2 (58). The motif repeats fused to the minimal reporter was sufficient to drive excretory cell expression of the reporter *in vivo*, albeit at a lower expression level than that the *aqp-8* promoter-reporter constructs, suggesting that there are other elements downstream of the octamer element that are important for fine-tuning the levels of mRNA production, but which themselves do not necessarily modulate the spatial pattern of AQP-8. This leads to a combinatorial model for gene expression of *aqp-8*. This is supported by multiple alignments of the region between the POU motif and the translational start site of four nematode species which show seven blocks of perfect conservation for sequences greater than six residues (Fig. 3). Another possible explanation for the lower observed GFP expression level as a result of the *Apes-10::GFP*-based construct is that the motif may have an optimal effectiveness at a specific distance relative to the translational start site. Further studies should be carried out to determine which if not both of these hypotheses are applicable in this situation. An alternative, osmotic balance controlled model of gene regulation has been also suggested for *aqp-8* (15). It is possible that some of the other conserved regions upstream of *aqp-8*'s may be responsible for this aspect of its regulation.

CEH-6's vertebrate ortholog is the class III POU homeobox protein, Brn1. Members of the class III POU transcription factors play important roles in the development of the nervous system (59). Zebrafish Brn1 has been localized via whole-mount *in situ* expression patterning to neuronal tissue (60). Brn1 expression has also been detected in the gastrointestinal tract of embryonic sea urchins (61). The Brn1 ortholog in quail has been observed, by whole mount *in situ* hybridization, to be localized in neuronal tissue and in the mesodermal sections of the developing kidney in day-5 old embryonic quail. In addition, it has been detected as early as day-2 old embryonic tissue sections by Northern Blot analysis (62). Homozygous mBrn1 deficient mice die within 24 hours of birth due renal complications. Dissection of two hour-old mice revealed that *mBrn1*^{-/-} mice had significantly lower volumes of urine compared to their wild-type

counterparts. mBrn-1 was observed to be localized to the macula densa, the distal convoluted tubule, and the Loop of Henle. Closer inspection of these tissues revealed a shortened loop of henle, and suppressed differentiation in all three tissues (63). The CEH-6 ortholog in the crustacean *Aretemia franciscana*, APH-1, is expressed in the salt gland, which like the *C. elegans* excretory cell and mammalian kidney, is an osmoregulatory organ. RT-PCR analysis of APH-1 reveals that, like CEH-6, the transcription factor is expressed predominantly during development (64). Since the excretory cell phenotype of *ceh-6(mg60)* manifests early in development (44) and due to the general role of POU homeobox TFs in modulating gene expression in early development we presume that many of the genes transcriptionally regulated by CEH-6 are required for morphogenesis of renal and neuronal tissues.

To determine genes which may be co-regulated with *aqp-8*, we searched for genes in which the octamer motif was perfectly conserved in the upstream region of three nematode species (*C. elegans*, *C. briggsae*, and *C. remanei*) and, using publicly available expression pattern data (29), we determined the frequency that the motif arises in the upstream region of excretory cell expressing genes. We did not observe a high level of significance when determining whether these genes were more likely than not to be expressed in the excretory cell. The low significance was likely caused by the lack of expression data for many of the genes predicted providing a small sample size. We expect that the significance level of the data would increase if expression pattern for a larger group of the bioinformatically predicted candidates were available. Upon pattern analysis of other genes in the most relaxed set (Table 2, all) we found 10 of the 19 expressing genes show expression in neuronal cells, a tissue that also expresses CEH-6 (Table 2).

Studies pertaining to transcriptional regulation in vertebrates can be difficult due to the lack of sequenced genomes, the tissue and physiological complexity of the systems, and problems with determining complete expression patterns due to long developmental time courses. The intergenic spacing in the *C. elegans* genome is

relatively compact; therefore studies of long-range regulation are usually not required for the identification of single-gene *cis*-regulatory elements, though long-range studies may identify islands of co-regulated gene clusters due to factors such as higher-order chromatin structure. In addition, many studies rely on expression profile correlations and/or determining over-representative motifs in the promoter-containing regions using whole-genome approaches. One of the problems of these expression pattern correlation studies is that tissue co-expression does not always imply gene co-regulation as we have shown in our study.

Though the experimentally identified octamer sequence was perfectly conserved and functional in the upstream regulatory region of *aqp-8*, the octamer motif did not necessarily drive excretory cell expression. Due to these results, we have concluded that the octamer motif, although probably a functional DNA region in the many cases in which it is perfectly conserved between nematode species, is not sufficient in all cases to drive expression in the excretory cell. We intend to study the expression pattern of the other candidate promoter regions to develop a better understanding of which tissues and at what frequencies the octamer motif modulates expression.

Since this work and the previous study by Zhao et al. 2005 were not exhaustive searches for *cis*-regulatory elements which modulate gene expression in the excretory cell, there are still other transcription factor binding sites which affect excretory cell expression. In this study, we have revealed a conserved relationship between a transcription factor and its cognate DNA binding locus which is relevant to both renal and neuronal development in nematodes and in other higher organisms.

REFERENCES

1. Nelson, F. K., Albert, P. S., and Riddle, D. L. (1983) *J Ultrastruct Res* **82**(2), 156-171
2. Buechner, M., Hall, D. H., Bhatt, H., and Hedgecock, E. M. (1999) *Dev Biol* **214**(1), 227-241
3. Buechner, M. (2002) *Trends Cell Biol* **12**(10), 479-484
4. Hedgecock, E. M., Culotti, J. G., Hall, D. H., and Stern, B. D. (1987) *Development* **100**(3), 365-382
5. Nelson, F. K., and Riddle, D. L. (1984) *J Exp Zool* **231**(1), 45-56
6. Gazzarrini, S., Kang, M., Epimashko, S., Van Etten, J. L., Dainty, J., Thiel, G., and Moroni, A. (2006) *Proc Natl Acad Sci U S A* **103**(14), 5355-5360
7. Denker, B. M., Smith, B. L., Kuhajda, F. P., and Agre, P. (1988) *J Biol Chem* **263**(30), 15634-15642
8. Agre, P., Preston, G. M., Smith, B. L., Jung, J. S., Raina, S., Moon, C., Guggino, W. B., and Nielsen, S. (1993) *Am J Physiol* **265**(4 Pt 2), F463-476
9. de Groot, B. L., and Grubmuller, H. (2001) *Science* **294**(5550), 2353-2357
10. Ishibashi, K., Sasaki, S., Fushimi, K., Uchida, S., Kuwahara, M., Saito, H., Furukawa, T., Nakajima, K., Yamaguchi, Y., Gojobori, T., and et al. (1994) *Proc Natl Acad Sci U S A* **91**(14), 6269-6273
11. Fu, D., Libson, A., Miercke, L. J., Weitzman, C., Nollert, P., Krucinski, J., and Stroud, R. M. (2000) *Science* **290**(5491), 481-486
12. Preston, G. M., Jung, J. S., Guggino, W. B., and Agre, P. (1993) *J Biol Chem* **268**(1), 17-20
13. Castle, N. A. (2005) *Drug Discov Today* **10**(7), 485-493
14. (1998) *Science* **282**(5396), 2012-2018
15. Huang, C. G., Lamitina, T., Agre, P., and Strange, K. (2007) *Am J Physiol Cell Physiol*
16. Kuwahara, M., Ishibashi, K., Gu, Y., Terada, Y., Kohara, Y., Marumo, F., and Sasaki, S. (1998) *Am J Physiol* **275**(6 Pt 1), C1459-1464
17. Kuwahara, M., Asai, T., Sato, K., Shinbo, I., Terada, Y., Marumo, F., and Sasaki, S. (2000) *Biochim Biophys Acta* **1517**(1), 107-112
18. Umenishi, F., and Verkman, A. S. (1998) *Genomics* **47**(3), 341-349
19. Rai, T., Uchida, S., Marumo, F., and Sasaki, S. (1997) *Am J Physiol* **273**(2 Pt 2), F264-273
20. Umenishi, F., and Verkman, A. S. (1998) *Genomics* **50**(3), 373-377
21. Kaldenhoff, R., Kolling, A., Meyers, J., Karmann, U., Ruppel, G., and Richter, G. (1995) *Plant J* **7**(1), 87-95
22. Brenner, S. (1974) *Genetics* **77**(1), 71-94
23. Hobert, O. (2002) *Biotechniques* **32**(4), 728-730
24. Thacker, C., Sheps, J. A., and Rose, A. M. (2006) *Cell Mol Life Sci* **63**(10), 1193-1204
25. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res* **25**(24), 4876-4882
26. Stein, L. D., Mungall, C., Shu, S., Caudy, M., Mangone, M., Day, A., Nickerson, E., Stajich, J. E., Harris, T. W., Arva, A., and Lewis, S. (2002) *Genome Res* **12**(10), 1599-1610
27. Frand, A. R., Russel, S., and Ruvkun, G. (2005) *PLoS Biol* **3**(10), e312
28. Rogers, S., Wells, R., and Rechsteiner, M. (1986) *Science* **234**(4774), 364-368
29. McKay, S. J., Johnsen, R., Khattra, J., Asano, J., Baillie, D. L., Chan, S., Dube, N., Fang, L., Goszczynski, B., Ha, E., Halfnight, E., Hollebakken, R., Huang, P., Hung, K., Jensen, V., Jones, S. J., Kai, H., Li, D., Mah, A., Marra, M., McGhee, J., Newbury, R., Pouzyrev, A., Riddle, D. L., Sonnhammer, E., Tian, H., Tu, D., Tyson, J. R., Vatcher, G., Warner, A., Wong, K., Zhao, Z., and Moerman, D. G. (2003) *Cold Spring Harb Symp Quant Biol* **68**, 159-169
30. Reboul, J., Vaglio, P., Rual, J. F., Lamesch, P., Martinez, M., Armstrong, C. M., Li, S., Jacotot, L., Bertin, N., Janky, R., Moore, T., Hudson, J. R., Jr., Hartley, J. L., Brasch, M. A., Vandenhaute,

- J., Boulton, S., Endress, G. A., Jenna, S., Chevet, E., Papatotiropoulos, V., Tolias, P. P., Ptacek, J., Snyder, M., Huang, R., Chance, M. R., Lee, H., Doucette-Stamm, L., Hill, D. E., and Vidal, M. (2003) *Nat Genet* **34**(1), 35-41
31. Li, H., Kamiie, J., Morishita, Y., Yoshida, Y., Yaoita, E., Ishibashi, K., and Yamamoto, T. (2005) *Biol Cell* **97**(11), 823-829
32. Ishibashi, K., Morinaga, T., Kuwahara, M., Sasaki, S., and Imai, M. (2002) *Biochim Biophys Acta* **1576**(3), 335-340
33. Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D. P., Zipperlen, P., and Ahringer, J. (2003) *Nature* **421**(6920), 231-237
34. Stein, L. D., Bao, Z., Blasiar, D., Blumenthal, T., Brent, M. R., Chen, N., Chinwalla, A., Clarke, L., Clee, C., Coghlan, A., Coulson, A., D'Eustachio, P., Fitch, D. H., Fulton, L. A., Fulton, R. E., Griffiths-Jones, S., Harris, T. W., Hillier, L. W., Kamath, R., Kuwabara, P. E., Mardis, E. R., Marra, M. A., Miner, T. L., Minx, P., Mullikin, J. C., Plumb, R. W., Rogers, J., Schein, J. E., Sohrmann, M., Spieth, J., Stajich, J. E., Wei, C., Willey, D., Wilson, R. K., Durbin, R., and Waterston, R. H. (2003) *PLoS Biol* **1**(2), E45
35. Blacque, O. E., Perens, E. A., Boroevich, K. A., Inglis, P. N., Li, C., Warner, A., Khattra, J., Holt, R. A., Ou, G., Mah, A. K., McKay, S. J., Huang, P., Swoboda, P., Jones, S. J., Marra, M. A., Baillie, D. L., Moerman, D. G., Shaham, S., and Leroux, M. R. (2005) *Curr Biol* **15**(10), 935-941
36. McCarroll, S. A., Li, H., and Bargmann, C. I. (2005) *Curr Biol* **15**(4), 347-352
37. Sturm, R. A., and Herr, W. (1988) *Nature* **336**(6199), 601-604
38. Klemm, J. D., Rould, M. A., Aurora, R., Herr, W., and Pabo, C. O. (1994) *Cell* **77**(1), 21-32
39. Finney, M., and Ruvkun, G. (1990) *Cell* **63**(5), 895-905
40. Sze, J. Y., and Ruvkun, G. (2003) *Proc Natl Acad Sci U S A* **100**(16), 9560-9565
41. Wang, L., and Way, J. C. (1996) *Mech Dev* **56**(1-2), 183-196
42. Greenstein, D., Hird, S., Plasterk, R. H., Andachi, Y., Kohara, Y., Wang, B., Finney, M., and Ruvkun, G. (1994) *Genes Dev* **8**(16), 1935-1948
43. Rose, K. L., Winfrey, V. P., Hoffman, L. H., Hall, D. H., Furuta, T., and Greenstein, D. (1997) *Dev Biol* **192**(1), 59-77
44. Burglin, T. R., and Ruvkun, G. (2001) *Development* **128**(5), 779-790
45. Jones, S. J., and Baillie, D. L. (1995) *Mol Gen Genet* **248**(6), 719-726
46. Ross, D. A., Magor, B. G., Middleton, D. L., Wilson, M. R., Miller, N. W., Clem, L. W., and Warr, G. W. (1998) *J Immunol* **160**(8), 3874-3882
47. Zhang, T. Y., Kang, L., Zhang, Z. F., and Xu, W. H. (2004) *Biochem J* **380**(Pt 1), 255-263
48. Gruber, C. A., Rhee, J. M., Gleiberman, A., and Turner, E. E. (1997) *Mol Cell Biol* **17**(5), 2391-2400
49. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) *Nature* **391**(6669), 806-811
50. Kennedy, S., Wang, D., and Ruvkun, G. (2004) *Nature* **427**(6975), 645-649
51. Zhao, Z., Fang, L., Chen, N., Johnsen, R. C., Stein, L., and Baillie, D. L. (2005) *J Biol Chem* **280**(46), 38787-38794
52. Perkins, L. A., Hedgecock, E. M., Thomson, J. N., and Culotti, J. G. (1986) *Dev Biol* **117**(2), 456-487
53. Mori, I., and Ohshima, Y. (1995) *Nature* **376**(6538), 344-348
54. Sulston, J. E., and Horvitz, H. R. (1977) *Dev Biol* **56**(1), 110-156
55. Sock, E., Enderich, J., Rosenfeld, M. G., and Wegner, M. (1996) *J Biol Chem* **271**(29), 17512-17518
56. Baranek, C., Sock, E., and Wegner, M. (2005) *Nucleic Acids Res* **33**(19), 6277-6286
57. Coates, J. C., and de Bono, M. (2002) *Nature* **419**(6910), 925-929
58. Brockie, P. J., Madsen, D. M., Zheng, Y., Mellem, J., and Maricq, A. V. (2001) *J Neurosci* **21**(5), 1510-1522

59. Spaniol, P., Bornmann, C., Hauptmann, G., and Gerster, T. (1996) *Nucleic Acids Res* **24**(24), 4874-4881
60. Hauptmann, G., and Gerster, T. (2000) *Dev Dyn* **218**(2), 345-358
61. Yuh, C. H., Dorman, E. R., and Davidson, E. H. (2005) *Dev Biol* **281**(2), 286-298
62. Lan, L., Liu, M., Liu, Y., Liu, Y., Zhang, W., Xue, J., Xue, Z., and He, R. (2006) *Dev Dyn* **235**(4), 1107-1114
63. Nakai, S., Sugitani, Y., Sato, H., Ito, S., Miura, Y., Ogawa, M., Nishi, M., Jishage, K., Minowa, O., and Noda, T. (2003) *Development* **130**(19), 4751-4759
64. Chavez, M., Landry, C., Loret, S., Muller, M., Figueroa, J., Peers, B., Rentier-Delrue, F., Rousseau, G. G., Krauskopf, M., and Martial, J. A. (1999) *Mech Dev* **87**(1-2), 207-212

FOOTNOTES

¹ The authors thank the members of our laboratories (DLB, HMC, and NSC) for productive discussions pertaining to this project. They would also like to thank the Mitani Laboratory for kindly providing the strain TM1919. A. K. Mah is supported by an NSERC doctoral scholarship. J. S. Chu is supported by a Weyerhaeuser-MBB fellowship and a Hemingway Nelson Architects Graduate Scholarship. H. M. Chamberlin is supported by the NSF grant IOB-0516554. N. S. Chen is supported by a grant from NSERC Canada and a Faculty start-up fund provided by Simon Fraser University. D. L. Baillie is a Canada Research Chair in Genomics and is supported by grants from NSERC and CIHR.

² Abbreviations: AQP, Aquaporin; GFP, Green Fluorescent Protein; CAT, chloramphenicol acetyltransferase; TESS, Transcriptional Element Search System; EMSA, Electrophoretic Mobility Shift Assay; PEST, degradation signal consisting of a Pro-Glu-Ser-Thr rich sequence; 3' UTR, 3' untranslated region; SAGE, Serial Analysis of Gene Expression; FACS, Fluorescence Assisted Cell Sorting; POU, (pit, oct, unc); NES, Nuclear Localization Signal; NES, Nuclear Export Signal.

TABLES

Table 1. List of *C. elegans* strains analyzed

Strain	Genotype
N2	N2 (Bristol) <i>C. elegans</i> wild type
CB907	<i>dpy-5(e907)</i>
BC07032	<i>dpy-5(e907);sIs1241 rCes[K02G10.7(-711)]::GFP-PEST +pCeh361</i>
BC20018	<i>dpy-5(e907);sEx20018 rCes[K02G10.7(-1.6kb)]::GFP-PEST + pCeh361</i>
BC20052	<i>dpy-5(e907);sEx20052 rCes[K02G10.7(-1.6kb)]::GFP + pCeh361</i>
TM1919	<i>aqp-8(tm1919)</i> kindly provided by S. Mitani
BC06835	<i>dpy-5(e907);sEx1241 rCes[K02G10.7(-711)]::GFP-PEST+pCeh361](SegI)</i>
BC06836	<i>dpy-5(e907);sEx1242 rCes[K02G10.7(-711)]::GFP-PEST+pCeh361](SegII)</i>
BC06837	<i>dpy-5(e907);sEx1243 rCes[K02G10.7(-567)]::GFP-PEST+pCeh361](SegI)</i>
BC06838	<i>dpy-5(e907);sEx1244 rCes[K02G10.7(-567)]::GFP-PEST+pCeh361](SegII)</i>
BC07228	<i>dpy-5(e907);sEx1525 rCes[K02G10.7(-434)]::GFP-PEST+pCeh361]</i>
BC07226	<i>dpy-5(e907);sEx1523 rCes[K02G10.7(-354)]::GFP-PEST+pCeh361]</i>
BC06839	<i>dpy-5(e907);sEx1245 rCes[K02G10.7(-342)]::GFP-PEST+pCeh361]</i>
BC07227	<i>dpy-5(e907);sEx1524 rCes[K02G10.7(-315)]::GFP-PEST+pCeh361]</i>
BC06876	<i>dpy-5(e907);sEx1278 rCes[K02G10.7(-297)]::GFP+pCeh361]</i>
BC06877	<i>dpy-5(e907);sEx1279 rCes[K02G10.7(-279)]::GFP+pCeh361](SegI)</i>
BC06878	<i>dpy-5(e907);sEx1280 rCes[K02G10.7(-279)]::GFP+pCeh361](SegII)</i>
BC06921	<i>dpy-5(e907);sEx1314 rCes[K02G10.7(-272)]::GFP+pCeh361](SegI)</i>
BC06922	<i>dpy-5(e907);sEx1315 rCes[K02G10.7(-272)]::GFP+pCeh361](SegII)</i>
BC06916	<i>dpy-5(e907);sEx1312 rCes[K02G10.7(-267)]::GFP+pCeh361]</i>
BC06879	<i>dpy-5(e907);sEx1281 rCes[K02G10.7(-261)]::GFP+pCeh361](SegI)</i>
BC06887	<i>dpy-5(e907);sEx1289 rCes[K02G10.7(-261)]::GFP+pCeh361](SegII)</i>
BC07229	<i>dpy-5(e907);sEx1526 rCes[K02G10.7(-228)]::GFP-PEST+pCeh361]</i>
BC06916	<i>dpy-5(e907);sEx1312 rCes[K02G10.7(-207)]::GFP+pCeh361](SegI)</i>
BC06840	<i>dpy-5(e907);sEx1246 rCes[K02G10.7(-207)]::GFP-PEST+pCeh361](SegII)</i>
BC06841	<i>dpy-5(e907);sEx1247 rCes[K02G10.7(-207)]::GFP-PEST+pCeh361](SegIII)</i>
BC06842	<i>dpy-5(e907);sEx1248 rCes[K02G10.7(-80)]::GFP-PEST+pCeh361](SegI)</i>
BC06843	<i>dpy-5(e907);sEx1249 rCes[K02G10.7(-80)]::GFP-PEST+pCeh361](SegII)</i>
BC06925	<i>dpy-5(e907);sEx1318 rCes[K02G10.7 (translational)]::GFP+pCeh361] (SegI)</i>
BC06926	<i>dpy-5(e907);sEx1319 rCes[K02G10.7 (translational)]::GFP+pCeh361] (SegII)</i>
BC06979	<i>dpy-5(e907);sEx1345 rCes[4xAATTTGCATA::Δpes-10::GFP+ pCeh361](SegI)</i>
BC07030	<i>dpy-5(e907);sEx1387rCes[4xAATTTGCATA::Δpes-10::GFP]+ pCeh361](SegII)</i>
BC07285	<i>dpy-5(e907);sEx1558 rCes[(<i>aqp-8 oct G</i> → <i>A</i>)]::GFP+pCeh361](SegI)</i>
BC07286	<i>dpy-5(e907);sEx1559 rCes[(<i>aqp-8 oct G</i> → <i>A</i>)]::GFP+pCeh361](SegII)</i>
BC07312	<i>dpy-5(e907);sEx1574 rCes[(<i>aqp-8 oct C</i> → <i>G</i>)]::GFP+pCeh361]</i>
BC10300	<i>dpy-5(e907);sEx10300 rCes[ZK742.1+pCeh361]</i>

Table 2. Filtering criteria used for determination of genes with conserved octamer motifs in their 1kb gene-upstream regions

Set	Filtering Criteria	Number of predicted genes	Number of genes with existing expression data	Number of excretory cell expression genes	Probability
All	No additional criteria	109	19	3	0.3556
S	Pou motifs are on the same strand as the downstream gene	54	13	3	0.1662
E	Pou motifs are not overlapping with any upstream gene	83	16	3	0.2623
SE	Pou motifs are on the same strand as the downstream gene and not overlapping with any upstream gene	44	10	3	0.0857

Table 3. Expression patterns of genes in *C. elegans* (lowest stringency) which have upstream octamer (ATTTGCAT) sites. The expression pattern data was taken from The Genome BC *C. elegans* Gene Expression Consortium webpage (http://elegans.bcgsc.ca/home/ge_consortium.html)

Sequence Name	Gene Name	Strain	Transgene ID	Expression Pattern
C01B12.5		BC20002	sEX20002	no expression.
C15B12.7	<i>cdf-1</i>	BC14040	sEX14040	no expression.
C16C10.4		BC14128	sEX14128	1st and 2nd pharyngeal bulbs; anterior neuron; posterior neuron; muscle; Intestine; vulva.
C26F1.10	<i>flp-21</i>	BC12205	sEX12205	Intestine; anterior neuron.
C38H2.1		BC13836	sEX13836	Nerve cord; anterior neuron; posterior neuron; amphids; nerve ring.
C45G9.5		BC12539	sEX12539	Intestine; excretory cell; anterior neuron; posterior neuron; vulva; hypoderm.
C54D10.1	<i>cdr-2</i>	BC15319	sEX15319	1st and 2nd pharyngeal bulbs; Vulva; Anterior neuron;
F14H12.1	<i>col-165</i>	BC16801	sEX16801	Hypoderm; seam cell.
F39H11.3	<i>cdk-8</i>	BC14622	sEX14622	no expression.
F53C11.3		BC14427	sEX14427	Intestine.
F53E2.1	<i>tag-304</i>	BC10230	sEX10230	Pharynx; muscle; anterior neuron; posterior neuron; vulva.
H24G06.1		BC15661	sEX15661	no expression.
H43I07.3		BC15071	sEX15071	1st and 2nd pharyngeal bulbs; Anterior neuron; Posterior neuron; Nerve ring; Pharynx;
K02G10.7	<i>aqp-8</i>	BC20052	sEX20052	Excretory cell;
R02F2.8		BC12904	sEX12904	no expression.
R08B4.2	<i>alr-1</i>	BC16630	sEX16630	no expression.
R10H1.2	<i>srab-14</i>	BC14834	sEX14834	Anterior neuron; nerve cord; excretory cell.
R13A5.1	<i>cup-5</i>	BC10182	sEX10182	Pharynx; muscle.
T05H10.3		BC14357	sEX14357	hypoderm.
T10B5.5		BC12510	sEX12510	1st and 2nd pharyngeal bulbs; nerve cord; nerve ring.
T12A2.9	<i>srg-8</i>	BC11603	sEX11603	Anterior neuron.
T14G10.5	<i>tsp-12</i>	BC13695	sEX13695	no expression.
T19A6.2	<i>ngp-1</i>	BC14682	sEX14682	Intestine; 1st and 2nd pharyngeal bulbs;
W08D2.1	<i>egl-20</i>	BC17158	sEX17158	Muscle; Anal depressor;
Y43F8C.12	<i>mrp-7</i>	BC10031	sEX865	Intestine; muscle; neuron.
Y54G2A.25	<i>lad-2</i>	BC13847	sEX13847	Nerve ring; posterior neuron.
Y7A9A.1		BC11932	sEX11932	no expression.
ZK512.9	<i>grl-11</i>	BC12881	sEX12881	no expression.

FIGURE LEGENDS

FIG. 1. GFP expression pattern analysis of AQP-8 *A*, The *aqp-8* 1.6kb upstream fragment drove GFP expression in the excretory cell (EC) and a secondary cell (♦) (L2). *B*, Expression of a transgenic strain carrying the *aqp-8p::GFP-PEST* construct. Spatial expression patterns of the *aqp-8p::GFP-PEST* containing strains were effectively identical to the expression of strains carrying promoter::GFP constructs. *C*, Expression of a stabilized transgene (genome-integrated tandem DNA array) leads to expression patterns that are identical to non-stabilized transgenic lines. *D*, Closer analysis of an adult worm expressing the AQP::GFP construct (translational reporter fusion) shows that the GFP is localized to the outer walls of the excretory cell canal. The expression level of the translational construct was slightly lower than that of the transcriptional constructs (L3). *E*, Expression of a stabilized transgene consisting of a 711bp upstream fragment fused to GFP-PEST. *All images were captured were at 400X magnification. **Camera conditions were the same for all images. ***Exposure times are as indicated on the GFP images.

FIG. 2. Deletional analysis of the *aqp-8* promoter region. The promoter region of *aqp-8* was truncated from the 5' end in an unbiased manner. When available, more than one strain line was assayed for transcriptional activity. Constructs with upstream regions depicted in black are further described in the text. Constructs with upstream regions depicted in grey are not mentioned in the text. GFP expression levels remained consistent until GFP expression was lost in the -261 lines. Since the -279 and -261 constructs were critical in this analysis, second independently isolated lines for each transgene were assayed to confirm their transcriptional activities.

FIG. 3. ClustalX alignment (default parameters) of the upstream regions of *aqp-8* orthologs (in the following order). *C. elegans*, *C. brenneri* (CB5161;contig 412), *C. briggsae*, *C. remanei*. All positions reported are in relation to the positions indicated in the alignment. The alignment reveals multiple blocks of perfect conservation in the promoter region. The most distal block, at position 141, (solid black box) contains the putative POU transcription factor binding motif. The arrows designated with 1 and 2 respectively indicate the -279 and -261 sites corresponding to the promoter-deletion constructs. Additional blocks of perfect conservation (>6 identical residues) are seen at positions: 224, 282, 291, 318, 326, 345, and 358 (dashed black boxes). The SL1 trans-splice site (TTTCAG), at position 431, is perfectly conserved in three out of the four nematode species. All sequences shown terminate at the -1 position relative to the translational start site of the gene.

FIG. 4. Validation of the putative *cis*-regulatory element. *A*, The ability of the promoter fragment to drive GFP expression in the *C. elegans* excretory system was abrogated when the terminal nucleotide of the octamer motif was excluded from the transgene construct. *B*, A -264 G→A mutation in the octamer site leads to an expression pattern identical to the original 1.6kb *aqp-8* promoter fragment, however, upon mutating the adjacent upstream residue in the octamer site (-263 C→G) led to complete loss of GFP expression (images not shown) *C*, A heterologous reporter construct consisting of four tandem repeats of (AATTTGCATA) fused to *Δpes-10::GFP* was sufficient to drive expression in an excretory cell specific manner. *D*, The *Δpes-10::GFP* construct expresses at a low level in the excretory cell in addition to the AUA and the AVH neuronal cell pairs.

FIG. 5. CEH-6 is required for *aqp-8::GFP* expression. Injection of dsRNA, corresponding to the *eri-1* gene, into AQP-8::GFP-expressing worms alone did not lead to a loss of GFP expression pattern in the excretory cell (top). Injection of two types of dsRNAs corresponding to *eri-1* and *ceh-6* leads to a consistent complete loss of GFP expression in the excretory cell of the AQP::GFP-expressing line

(bottom). *Images are of L2 larval *C. elegans* 48-hours post dsRNA injection **Camera conditions and exposure times were the same for all images.

FIG. 6. Electrophoretic mobility shift assay. The DNA fragment consisting of 5'-ATTGCCAAAATTTGCATACTGGAAT - 3' and its complement 5' - ATTCCAGTATGCAAATTTTGGCAAT - 3' were incubated *C. elegans* cytosolic and nuclear protein extracts *in vitro*. *A*, The labelled probe was able to bind nuclear proteins *in vitro*. A greater degree of protein binding was observed when the probe was incubated with cytosolic protein extracts. The specificity of the octamer motif for the nuclear binding protein was determined by competitive binding of 1000X molar excess of unlabeled competition probe. In the presence of excess unlabelled probe, loss of protein binding to the labelled probe was observed in both the nuclear protein fraction. *B*, The specificity of the octamer motif for the cytosolic binding protein was determined by competitive binding of 1000X molar excess of unlabeled competition probe. In the presence of excess unlabelled probe, loss of protein binding to the labelled probe was observed in the cytosolic protein fraction. The dividing line (white) between the cytosolic and cytosolic + 1000XS lanes indicates that two lanes are from different parts of the same gel.

Fig1

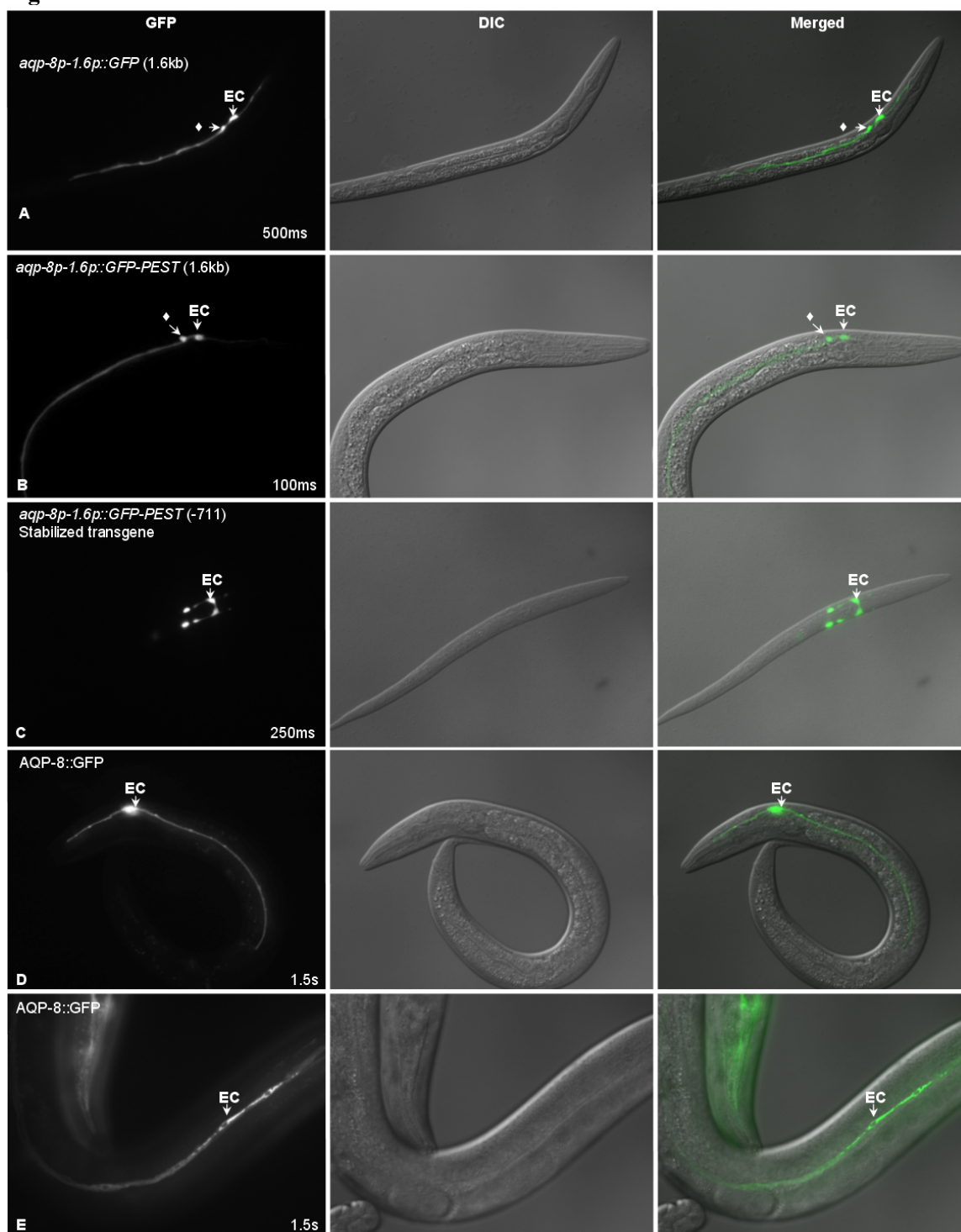


Fig2

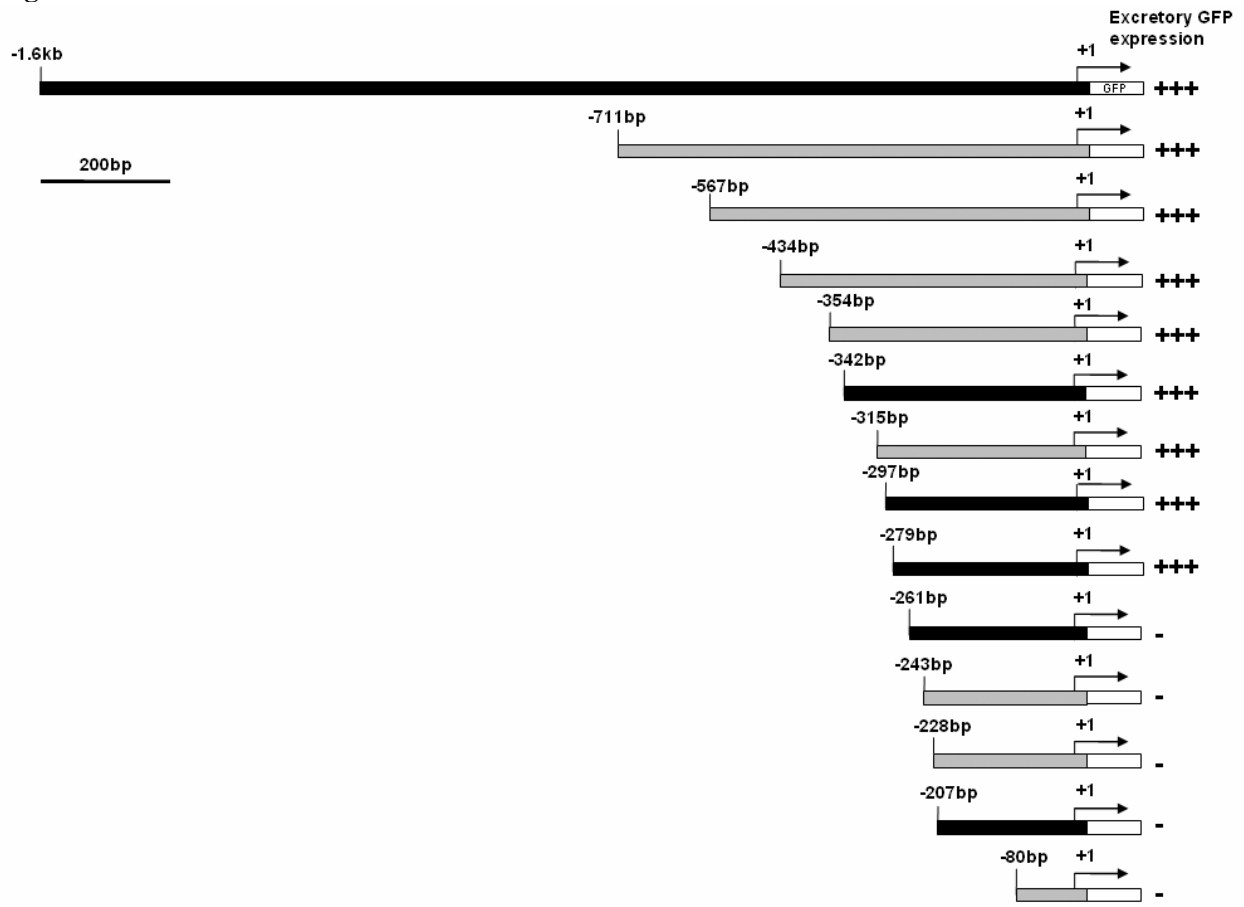


Fig3

```

elegans : TGAAAATGCCAAAGTCATTTAGACACATTA-GCTAATAG----CCATGAAATCTAACCTGAGCACCC : 68
Contig412. : --AAAATGTCTGAGTAACTTCAATCTAATATACCCCAAGATCCGGTGAACCTTATTCTATGTCGCC : 72
briggsae : -----TTACGATTTTCAGAAATTA-TCCATTGTG--TGTCCAGAAATTTTATCCCTCCGGT : 57
remanei : AGTCTTCGGTGAATTAATGTTTCAGAGA-ACCTGACAGAAAGTT--CCCTGAAACCTTTTGGT---TTATT : 68
          t      a      atTT a A a A T A g t t      c      gAA Ttt t      t c c

          80          *          100          *          120          *          140          *
elegans : TAAATGTTTATAGTAGTGTATATGATTCAGTGTGAATGCGCTAACCGCAAAA-TGATTGCCAFAATTGCATA : 141
Contig412. : GTTAAACATTATGCAC---ACATGATTCATG--TAAACCGTTTGC-CATGGTTCAGTGTGCAFAAATTGCATA : 140
briggsae : AGACAGTGACACATTTGICATGCATGATTCACGGTGAAGTAAAAATTGAC---AACTCTGAFAATTGCATA : 127
remanei : AGACAGTGAACACTGCACATATG--TCATGACATGACTGAGTAAACCATG---AAACTGGAFAATTGCATA : 135
          a a t t      G      a ATGatTCA g t gaa t t c      a      c FAATTGCATA

          *          160          *          180          *          200          *          220          *
elegans : CTG---GAATTGCG---AGTCAATCA-AGCTGGATTATGATAAGTACTGCCAAACCGAA--AACTTTTCA- : 204
Contig412. : TTG---AAACTG---AGTCAATCA-AGCTGGATTATGATAAGTATTCCTATTTGAAACCGAAGCAAAAA- : 203
briggsae : GAATTGABAATTCGGTCCGGATTATCATACTATACAGTTTTTCABAATTTATCAACCGA-AACCGCATAAACT : 200
remanei : CCA---AACTCAATTGACAGCATCA-ACCAGGATTAATTAAGTATTTCTATTTTGAACCGGTGA--- : 199
          AA T      aG c ATCA A c gGattaT aTaAgTatT tCa      cAAccga aa

          *          240          *          260          *          280          *
elegans : CCGCGCT---TGT-TTATCTATTTTCGTCCTAC---TAGAATATCAGGGCTACCGTAACATCCCGG : 267
Contig412. : CCGCGCT---CAATGTATTCGTATCTTCTCTAC---TTTG-TGTCACCGCTACCGTAACATCCCGG : 266
briggsae : CCGCGCTTCAAATCAAGCATTCATCAATCTCATCAAAATTTTCTTCTACCCCTACCGTAACATCCCGG : 274
remanei : CCGCGCT---CGGACGATTCGTATCTTCTTAC---TTTGTGTCACCGCTACCGTAACATCCCGG : 261
          CCGCGCT      a g ATtcgtaTcttcTctTaG      ttt TgTCACCGCTACCGTAACATCCCGG

          *          300          *          320          *          340          *          360          *
elegans : CCATTGTGAAGTTCGGCACTGTCCCTTCCCTATTTAAAAATAGCG-CACGCCCTCTGATAAGGACATTTC : 340
Contig412. : CCATTGTGAAGTTCGGC-STGTCCCTTCCCTATTTAAAAATAGCG-CACGCCCTCTGATAAGGACATTTC : 338
briggsae : CCATTGTG-----CG-STGTCCCTTCCCTATTTAAAAATAGCG-CACGCCCTCTGATAAGGACATTTC : 340
remanei : CCATTGTGAAGTTCGGC-STGTCC-STGTCCCTTCCCTATTTAAAAATAGCG-CACGCCCTCTGATAAGGACATT : 333
          CCATT-TGAagtt gcc STGTCC TTTCTATTTAAAAAT AGCG CACGCCCTCTGATAAGGACATT T

          *          380          *          400          *          420          *
elegans : CGTCCATACCATTTCTTTT---TTTGTGCAT--CCGTTCCTATAAATTTATTTATTTTATAGATA : 401
Contig412. : CGTCCGTGATATGTTGTTTC---CGGTAGGGTGCCTAATTTATGCTTTATTTTTCAGATA : 401
briggsae : CGTCCACGTTGTTTTCCTCAAGAAATTTTC---TTTATTTTACT---AATTTATTTTCAGATA : 401
remanei : CGTCCGCCACCTTCTTTTCTTTTAAATCTTAGCAGATTTATTTCAATGCGAGATTTTCAGGAT : 401
          CGTCC t t TT T Tt t      tt T gg      t aTTtta t t ta ttaTTTTcAGaTa
    
```



Fig4

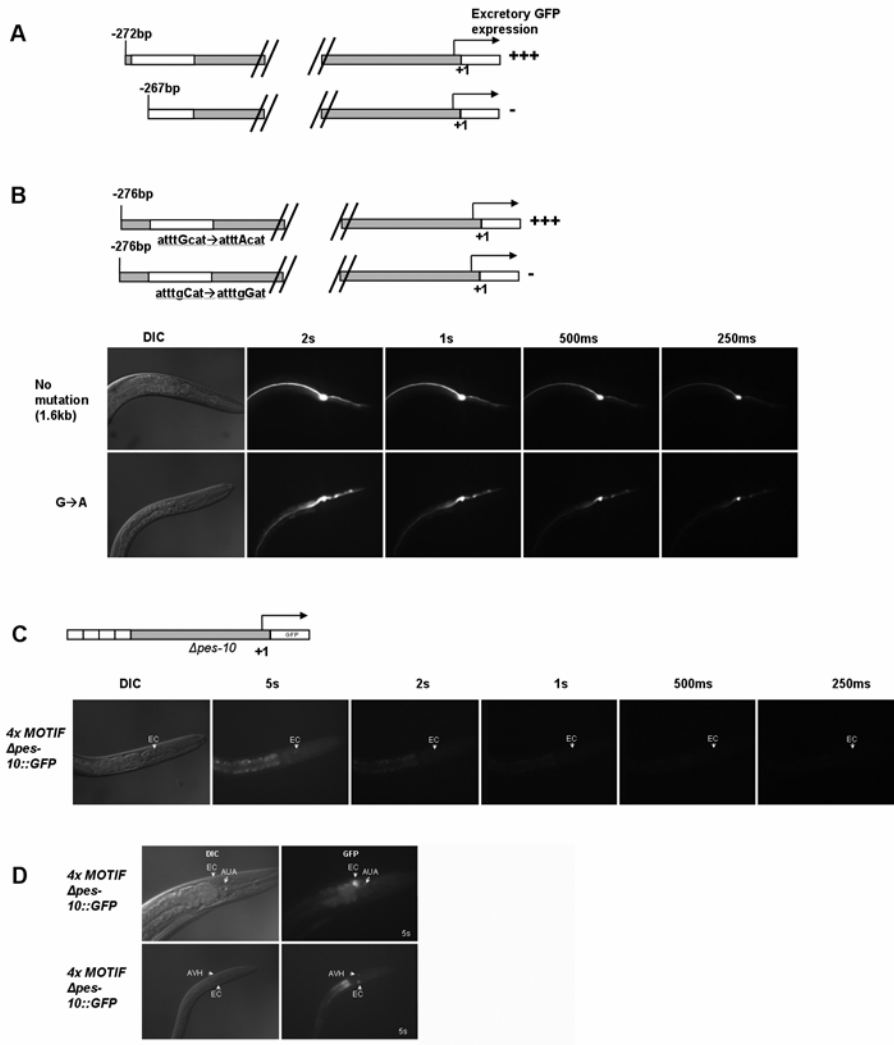


Fig5

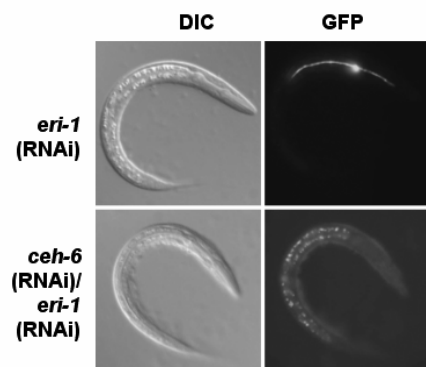


Fig6

