

*Letter to Neuroscience*

EVOLUTION TUNES THE EXCITABILITY OF INDIVIDUAL NEURONS

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MO 63110, USA^bDepartment of Genetics, Washington University School of Medicine, 660 South Euclid, St Louis, MO 63110, USA*Key words:* potassium channels, genes, TWIK, task, *C. elegans*, evolution.

The relationship between the genome and the evolution of the nervous system may differ between an animal like *C. elegans* with 302 neurons, and mammals with tens of billions of neurons. Here we report that a class of nonconserved potassium channels highly expanded in *C. elegans* may play a special role in the evolution of its nervous system. The *C. elegans* genome contains an extended gene family of potassium channels whose members fall into two evolutionary divergent classes. One class constitutes an ancient conserved “set” of K⁺ channels with orthologues in both humans and *Drosophila*^{1,24} and a second larger class made up of rapidly evolving genes unique to *C. elegans*.²⁴ Chief among this second class are novel potassium channels having four transmembrane domains per subunit^{6,8,14,20} that function as regulated leak conductances to modulate cell electrical excitability. This inventory of novel potassium channels is far larger in *C. elegans* than in humans or *Drosophila*. We found that, unlike conserved channel genes, the majority of these genes are expressed in very few cells. We also identified DNA enhancer elements associated with these genes that direct gene expression to individual neurons. We conclude that *C. elegans* may maintain an exceptionally large inventory of these channels (as well as ligand-gated channels¹) as an adaptive mechanism to “fine tune” individual neurons, making the most of its limited circuitry. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

“TWK” potassium channels [potassium channels with two ‘P’ (pore-forming) domains per subunit] constitute

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Abbreviations: DVA, interneuron with cell body in dorsal rectal ganglion and process extending to nerve ring; EST, expressed sequence tag; GFP, green fluorescent protein; RIG, ring interneuron G; TASK, a mammalian TWK potassium channel subfamily; TWK, potassium channels with two “P” (pore-forming) domains per subunit.

more than half of the K⁺ channels in *C. elegans*.^{1,8,24} While there are more than 40 TWK-encoding genes in *C. elegans*, there are only 11 in *Drosophila*.^{15,19} Some of this difference in number may be accounted for by alternative RNA splicing in *Drosophila*, but TWK genes are also alternatively spliced in *C. elegans*.²² There are also several examples of very recent gene duplications in *C. elegans* (G. Paz-Y-Mino, G. Fawcett and L. Salkoff, unpublished observations). Extrapolation from the partially completed human database suggests that the human genome contains approximately one third the number of TWK genes present in *C. elegans*. We estimated the relative expression levels for TWK- and other potassium channel-encoding genes in *C. elegans* by analyzing their representation in the public *C. elegans* expressed sequence tag (EST) database.^{7,12} While genomic sequence represents genes irrespective of their expression levels, an EST library is biased towards more highly expressed genes. In general we found that members of the conserved ‘set’ of potassium channel orthologues, like those previously mentioned, were well represented in the EST database. However, 31 of 41 TWK channels were not represented (Table 1). The public *C. elegans* EST library⁷ originates from cDNA synthesized from the entire animal. Thus, higher representation in the EST library may mean that a particular gene is expressed in a larger number of cells or tissues in the animal. Conversely, lack of representation could mean a more restricted cell-type expression pattern. To investigate whether a lack of representation for many TWK genes would correlate with a restricted expression pattern, we undertook promoter-green fluorescent protein (GFP) transformation experiments for genes both represented and absent in the EST database. Table 1 divides the TWK channel genes into two categories: those represented in the EST database (10 genes), and those not represented (31 genes). Promoter-GFP transformation experiments were completed for four of the 10 represented genes, and 16 of 31 genes not represented. Results

Table 1. Potassium channels in *C. elegans* having two pore regions per subunit

Channel	Cosmid	GB accession	No. ESTs	EST's (16,17)	Expression pattern	Notes
TWK channels represented in the EST database						
n2P31	Y47D3B.5	AL031635	5	yK19g4 5'3';yk502a9 5'yk449e10 5'3'; yk149d7 5';yk548a6 5'3'		
n2P8	ZC410.4	Z68270	4	yk2c7 5'3';yk590d7 5'3';yk128h9 5'3';yk195d 5'3'	Body-wall muscle (Fig. 1a)	
n2P11	F20A1.7	U53150	3	yk9g1 5'3';CEESU55;yk288el 5'		
n2P15	F32H5.2	Z81524	2	yk240f2 5'3';yk354h3 5'3'		
n2P18	C24A3.6	U40424	2	yk305h4 5'3';yk411b8 5'3'	Body-wall muscle (Fig. 1a)	
n2P5	B0334.2	Z66519	1	yk95h12 5'3'		
n2P9	ZK1251.8	Z68222	1	cm18h8 5'		
n2P19	T06H11.1	Z49889	1	yk33h6 5'	Motor neurons & interneurons (Fig. 1a)	
n2P28	C52B9.6	U64598	1	yk467b11 5'3'	Body-wall muscle	
n2P33	W06D12.5	Z82073	1	yk224a2 5'3'		
TWK channels not represented in the EST database						
n2P1	F21C3.1	Z71261	0	0	Hypoderm	
n2P2	T12C9.3	U41104	0	0	Subset of neurons	
n2P3	M110.2	Z49968	0	0	Subset of neurons (Fig. 1b)	Pharynx in early stages
n2P4	ZK1067.5	Z70038	0	0	Subset of sensory neurons (Fig. 1b)	
n2P6	F17C8.5	Z35719	0	0	Hypoderm	
n2P7	F22B7	L12018	0	0		
n2P10	K04A8 (twk-24)	U64849	0	0		
n2P12	F29F11.4	Z73974	0	0		
n2P13	R04F11.4	Z74475	0	0		
n2P14	K01D12.4	Z75543	0	0		
n2P16	F52E4.4	U56964	0	0	Subset of interneurons (Fig. 1b)	
n2P17	C44E12.3	U39647	0	0	Subset of neurons	Alternative splicing (15)
n2P20	C40C9 (twk-4)	Z70266	0	0	Neurons & muscle	Orthologue of TASK1(4)
n2P21	T01B4.1	Z70036	0	0		
n2P22	T01B4.2	Z70036	0	0	Pharynx (Fig. 1b)	
n2P23	F19D8.1	Z78541	0	0	Neurons, muscle, posterior intestine	Diffuse in many tissues
n2P24	F55C5.3	Z78198	0	0		
n2P25	M04B2.5	Z77667	0	0		
n2P26	C33D12.3	U64600	0	0		
n2P29	F46A9.3	Z81084	0	0	Subset of interneurons	
n2P30	F36A2.4	Z81077	0	0	Subset of motoneurons	
n2P32	F53C11.6	Z79756	0	0	Subset of interneurons (Fig. 1b)	
n2P34	K06B4.12	Z83233	0	0		
n2P35	F31D4.7	Z92832	0	0	Subset of motoneurons	
n2P36	R12G8.2	Z93782	0	0	Excretory cell (Fig. 1b)	
n2P37	C48E7.9	AF000262	0	0		
n2P38	F34D6 (twk-23)	AF025454	0	0	Neurons & muscle	Orthologue of TASK3
n2P39	C24H11.8	Z81475	0	0		
n2P40	T28A8.1	Z92813	0	0		
n2P41	Y37A1B.11	AL023835	0	0		
n2P42	Y76A2	Z92866	0	0		

C. elegans TWK channels are designated n2P1 through n2P42. The EST's listed are labeled as having been sequenced from their 5' or 3' ends, or both. The public *C. elegans* EST library consisted of approximately 30,000 independent EST sequences (as of 7/01/00). In this organism of approximately 19,000 genes, only the more abundantly expressed genes are represented in an EST library of this size.

are shown in Fig. 1. Figure 1 (+EST's) shows expression patterns for represented genes. TWK channels in this class were either expressed in all body-wall muscle (n2P8, n2P18, n2P28) or in many neurons (n2P19) (Fig. 1 +EST's). Thus, as hypothesized, representation in the EST database appears to be the result of expression in a larger number of cells. In contrast, TWK channels not represented in the EST database usually were expressed in fewer cells, and expression was often limited to small groups of neurons (Table 1; Fig. 1 -EST's). Restricted expression patterns for these TWK

genes included subsets of chemosensory neurons (n2P4), mechanosensory neurons (n2P3), interneurons (n2P3, n2P16, n2P32, n2P29) and motor neurons (n2P30). Expression of TWK genes was not limited to neurons. n2P36 is expressed in the single giant H-shaped excretory cell (Fig. 1, -EST's, n2P36; note the single large nucleus). n2P22 is expressed in the pharynx. Other TWK genes are expressed in hypoderm (n2P1, n2P6). An exception to the generally restricted pattern of TWK channels lacking EST's was the pattern of expression for the two orthologues of the TASK family (a

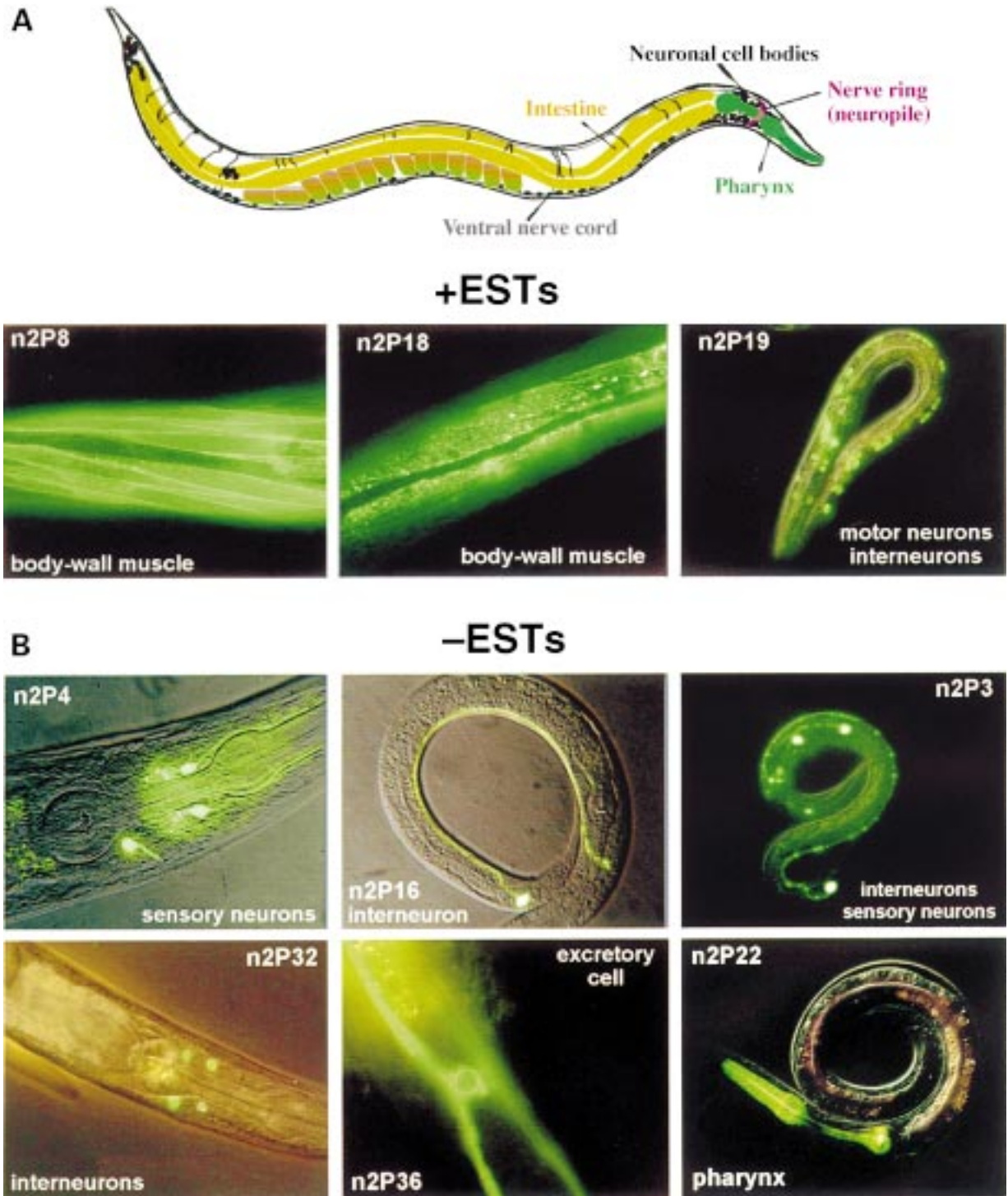


Fig. 1. TWK potassium channel gene expression in *C. elegans* visualized by Promoter-GFP transformation experiments. (A) Expression patterns of genes represented in the EST database. The row of panels shows three TWK channels, n2P8, n2P18, n2P19 represented by 4, 2, and 1 EST, respectively (see text for further description). (B) Restricted expression patterns of genes not represented in the EST database. Panels showing neuronal cell bodies without processes are from vector constructs containing a nuclear localization signal. In n2P32, cell bodies shown are a composite from two focal planes.

mammalian TWK channel subfamily), n2P20 and n2P38 (Table 1). These two channels had wide but faintly detected expression patterns, with expression present in muscle and a variety of neurons.

An animal with so few neurons can afford to assign a

unique genetic identity to each cell. Hence, we wondered whether TWK genes with limited expression patterns contained unique DNA enhancer elements responsible for directing gene expression to individual cells. Two genes were chosen to study this question, n2P16 and

n2P3. n2P16 had robust expression in the DVA neuron (interneuron with cell body in dorsal rectal ganglion) which is distinctive for its morphology, having a cell body in the tail and a long process extending to the

nerve ring (Fig. 1, -EST's, n2P16). Thus, we sought to identify the DNA sequence of the enhancer that selectively directed gene expression to this single cell. This study was aided by the availability of the complete

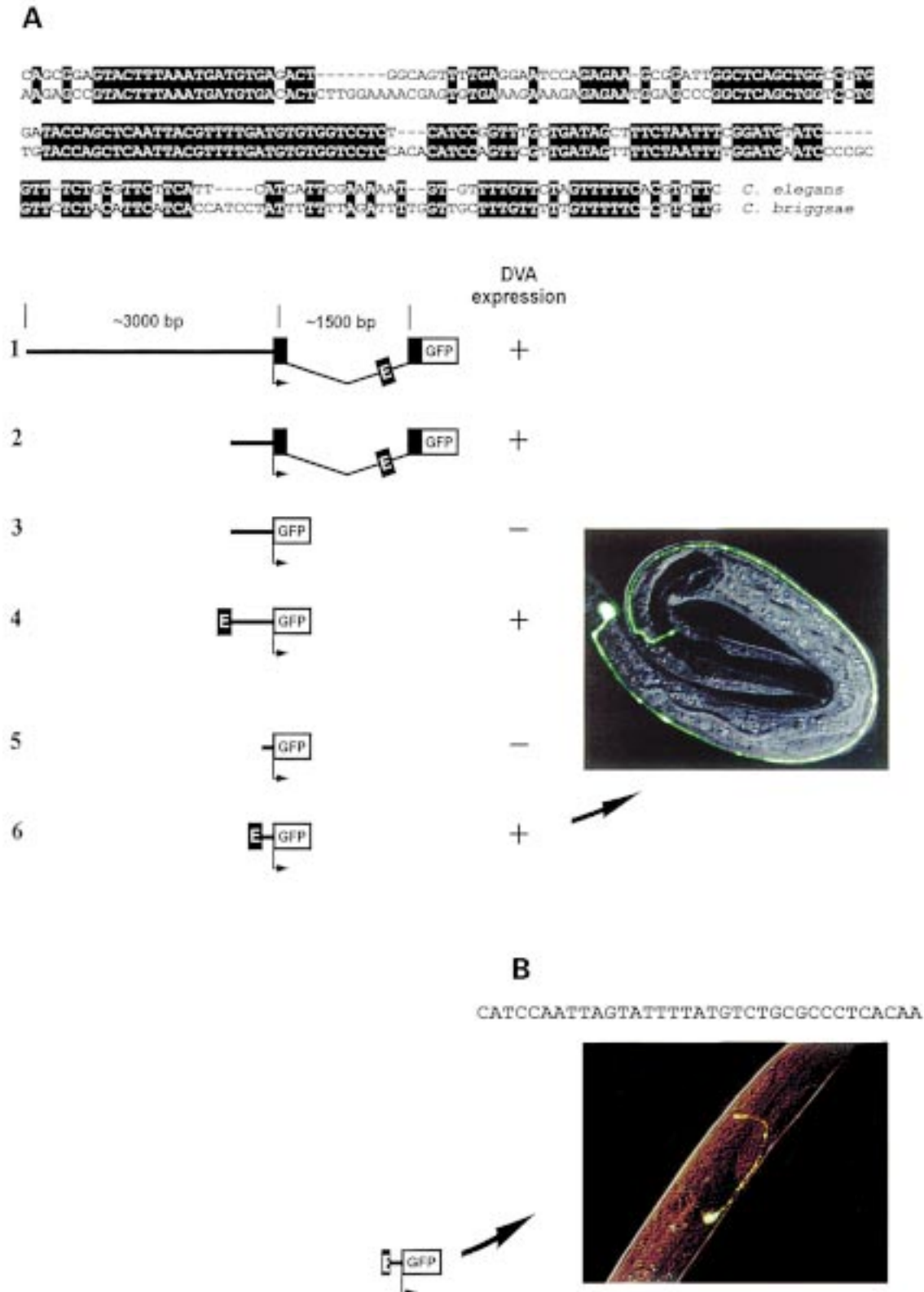


Fig. 2.

genomic sequence of the n2P16 orthologue in a closely related nematode species, *C. briggsae* (DNA sequence provided by the Genome Sequencing Center at Washington University). A comparative alignment of the entire gene sequence from the two nematode species permitted identification of conserved regulatory regions. The two genes were co-linear along their entire length and coding regions were easily identifiable.²² Outside of the n2P16 coding region, DNA sequence identity averaged less than 50% but islands of conspicuously high DNA sequence identity pointed to regions of important regulatory function. The region of highest conservation was found in the first intron. This region contained a block of 34 identical residues within a 213 base-pair segment (Fig. 2a). To investigate whether this sequence contained a neuron specific enhancer we transformed animals with a series of promoter-GFP transformation vectors that placed this 213 base-pair segment at different locations relative to the promoter and translation start site; in all instances it directed gene expression to the DVA neuron (Fig. 2a). Furthermore, even combined with a minimal promoter from a different gene, this 213 base-pair segment directed high-level gene expression exclusively to the DVA neuron (Fig. 2a, construct 6 and color micrograph).

A similar neuron-specific enhancer was found in n2P3 (Fig. 2b). n2P3 is expressed in a subset of neurons that includes the RIG ring interneurons. By a series of deletions we isolated a 35 base-pair segment that directs gene expression exclusively to the RIG (ring interneuron G) interneurons (Fig. 2b). In contrast to the DVA-specific enhancer which was located in an intron, the RIG-specific enhancer was located immediately upstream from the native n2P3 promoter. Other cell-specific enhancers are located further upstream from the RIG enhancer (not shown).

A blast search using the DVA enhancer sequence as query failed to find a conserved copy associated with any other gene in the *C. elegans* genome. Similarly the RIG enhancer sequence from n2P3 is present only once in the genome. Both of these enhancer elements that drive neuron-specific gene expression are apparently distinct from more typical regulatory sequences in a eukaryotic genome which have motifs that are shared by sets of functionally related genes.³ This pairing of unique cell-specific enhancer sequences with genes encoding unique potassium channel types may allow the associated ion

channel to be tailored by evolution to fit the needs of individual or small groups of neurons, without affecting other neurons or cell types in the animal.

Why does *C. elegans* with such a 'simple' nervous system require so many unique potassium channels? Among the 80 or so genes encoding potassium channels in *C. elegans* are those that encode conserved channel proteins that have greater amino acid conservation with their orthologues in higher species than they have with other potassium channels in the same species (paralogues). Examples of such genes are *Slo1*, the gene encoding the high conductance calcium-activated K⁺ channel which, in mammals, is expressed in both the brain and various muscle types,^{4,10} *KvLQT*, mutations of which in humans cause cardiac arrhythmia,²¹ and members of the voltage-dependent K⁺ channels: Shaker (Kv1), Shaw (Kv3) and Shal (Kv4)^{9,18,23} which, in mammals, are widely expressed in brain and other tissues. These ancient, highly conserved genes apparently arrived at their present forms in a common ancestor to most metazoan life and appear necessary for the proper functioning of all nervous systems. However, genes encoding TWK potassium channels are less conserved and seem to have a more recent origin, resulting from ongoing species-specific duplication and divergence. The expanded number of these genes in *C. elegans* at first glance seems at odds with the needs of such a 'simple' animal having so few cells. However, even though *C. elegans* hermaphrodites have only 302 neurons, they can be assigned to 118 distinct classes based on the details of morphologies and connections.^{25,26} Such heterogeneity suggests complexity (consider the mammalian cerebellum with approximately 10 billion cells but only five classes of neurons). A case has been made that neurons are not merely elements of circuitry, but complex computing devices in their own right.¹¹ This may be especially true in *C. elegans* where single neurons may serve the functional roles of millions of neurons (or even an entire brain region) in a higher animal. Perhaps in an animal with so few neurons evolution continually fine-tunes and adapts each neuron as an alternative strategy to amplifying circuitry. The large inventory of nematode-specific potassium channels may simply represent the tools of this ongoing optimization. Significantly, not only is the inventory of potassium channels unusually large in *C. elegans*, the inventory of

Fig. 2. DNA enhancer regions that confer gene expression in single neurons. (A) DVA enhancer region from n2P16. Comparative alignment shows the 213 base-pair region containing the DVA enhancer from *C. elegans* (top sequence), and *C. briggsae* (bottom sequence). Black shaded residues indicate identity with *C. briggsae*. The conserved sequence is located in the same position within the first intron in both species. Six vector constructs used in transformation experiments are diagrammed below the sequence. Constructs one through four are with the native promoter from n2P16. Constructs five and six are with the minimal promoter from n2P3. The enhancer region is designated 'E'. Animals transformed with constructs one, two, four and six all show DVA expression. In addition to its expression in the DVA, n2P16 is also expressed in an additional unidentified neuron in the nerve ring. Expression in this additional neuron was present with constructs one and two, but not with constructs four and six where expression was exclusively in the DVA. In construct four, the 231 base-pair conserved segment was ligated 500 base-pair upstream from the initiator methionine of n2P16, and all downstream coding and intronic sequence of n2P16 was replaced with that of GFP. In construct six the DVA enhancer region was ligated upstream from the 115 base-pair minimal promoter from n2P3; and as in four, GFP replaced all channel sequence. The color micrograph showing DVA-specific GFP fluorescence is from an animal transformed with construct six. (B) 35 base-pair RIG enhancer from n2P3. The RIG ring interneurons are a subset of neurons, which express n2P3 (Fig. 1b). The color micrograph showing RIG-specific GFP fluorescence is from an animal transformed with a vector containing the minimal promoter from n2P3 and the 35 base-pair enhancer sequence (E). This construct is similar to that of construct six in A, except that the DVA enhancer has been replaced with the 35 base-pair RIG enhancer. A second labeled RIG interneuron is out of the plane of focus.

ligand gated ion channels is unusually large, as well.¹ This could likewise be the result of the same evolutionary pressures. What functions might be supplied by TWK potassium channels? Several studies with both mammalian^{6,14,17,20} and *C. elegans* TWK channels¹³ indicate that these channels may provide a regulated leak current modulated by a multitude of factors including temperature,^{13,17} mechanosensation,¹⁶ pH⁶ and neurotransmitters.²⁰ Recently it was shown that in mammalian cerebellar granule cells an increase in a 'leak' conductance with properties characteristic of the two-pore-domain K⁺ channel TASK-1, can compensate for the loss of tonic inhibition mediated by GABA_A receptors to maintain normal neuronal behaviour.²

In *C. elegans*, the selective pressures that keep the nervous system low in cell number may be at work with other organ systems as well; adult hermaphrodites have a total of less than 1000 cells. Since most cells in the animal (neurons, muscle, hypoderm, pharynx, intestine) express a TWK channel, it appears as if *C. elegans* has discovered that a TWK channel can supply an adaptive advantage to virtually any cell type. Thus, these extra genes may permit *C. elegans* to continually optimize the properties of each member of its limited cell population in a multitude of ways. In mammals, it is of course not feasible to treat each neuron as a genetically discrete unit and assign a unique gene to each. Perhaps brain evolution in mammals more heavily involves the tinkering of control regions of existing genes to direct the amplification of neuronal circuitry. This may partially address a puzzle raised by the recent completion of the human, fly, and worm genomic databases which has revealed a somewhat shockingly low

number of genes in humans relative to the nematode worm.

EXPERIMENTAL PROCEDURES

To visualize the sites of potassium channel gene expression vectors were designed so that the native gene promoter would drive expression of a channel-GFP fusion construct. Vectors were created by ligating the gene for GFP in-frame to a coding portion of a genomic clone. Fluorescence is visualized directly in living animals.⁵ Generally the entire upstream region extending 5' to the proximal upstream gene was included. Upstream genes were predicted by a genefinder program on the designated cosmids (Table 1) and gene maps showing their predicted locations are available from NCBI. However, when the next upstream gene was further than approximately five kilobases, a transformation technique was used that included an entire cosmid containing the complete gene and 5' region. In this technique, recombination is induced between a subcloned genomic fragment and the designated cosmid. Details of this technique have been previously published.²⁷

The genes chosen for these expression studies are representative, and in all attempted expression experiments, except one, (n2P21) experimental outcomes were positive (the upstream region of n2P21 contains many tandem repeats and vector constructs are unstable). Experiments to determine the expression patterns of the remaining genes are ongoing and statistical evidence suggests that virtually all will reveal expression patterns. It should be noted that a positive GFP signal shows that, not only is the RNA transcribed, but the protein is made as well. Where we have looked for rare cDNA's by reverse transcription-polymerase chain reaction (seven instances) we have found them. Thus, we have found no evidence that any of the genes listed in Table 1 are pseudogenes. Further information concerning the genes described in this publication can be obtained from <http://nt-salkoff.wustl.edu>.

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