Functional tests of enhancer conservation between distantly related species

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Summary

Expression patterns of orthologous genes are often conserved, even between distantly related organisms, suggesting that once established, developmental programs can be stably maintained over long periods of evolutionary time. Because many orthologous transcription factors are also functionally conserved, one possible model to account for homologous gene expression patterns, is conservation of specific binding sites within cis-regulatory elements of orthologous genes. If this model is correct, a cis-regulatory element from one organism would be expected to function in a distantly related organism. To test this hypothesis, we fused the green fluorescent protein gene to neuronal and muscular enhancer elements from a variety of *Drosophila melanogaster* genes, and tested whether these would activate expression in the homologous cell types in *Caenorhabditis elegans*. Regulatory elements from several genes directed appropriate expression in homologous tissue types, suggesting conservation of regulatory sites. However, enhancers of most *Drosophila* genes tested were not properly recognized in *C. elegans*, implying that over this evolutionary distance enough changes occurred in cis-regulatory sequences and/or transcription factors to prevent proper recognition of heterospecific enhancers. Comparisons of enhancer elements of orthologous genes between *C. elegans* and *C. briggsae* revealed extensive conservation, as well as specific instances of functional divergence. Our results indicate that functional changes in cis-regulatory sequences accumulate on timescales much shorter than the divergence of arthropods and nematodes, and that mechanisms other than conservation of individual binding sites within enhancer elements are responsible for the conservation of expression patterns of homologous genes between distantly related species.

Supplemental data available online

Key words: Evolution, Enhancer, *C. elegans*, *C. briggsae*, *D. melanogaster*, Co-evolution

Introduction

Key developmental regulators and their expression patterns are conserved across a wide range of taxa (Carroll et al., 2001; Davidson, 2001). However, it is not yet clear what molecular mechanisms maintain similar expression patterns of homologous genes in different organisms, sometimes for over half a billion years. Classical experiments suggested that evolution at the regulatory level is largely responsible for the morphological changes observed in nature (Wilson et al., 1974; King and Wilson, 1975). This notion is strongly supported by the observation that even distantly related organisms use similar sets of basic developmental programs and it is the redeployment and subtle modification of these programs that generates morphological diversity. Recently, morphological differences between closely related species have been attributed to specific changes in *cis*-regulatory elements (Skaer and Simpson, 2000; Sucena and Stern, 2000). Empirical observations (Ludwig et al., 1998; Ludwig et al., 2000) and computer simulations (Stone and Wray, 2001) indicate that sequences of enhancer elements evolve relatively rapidly, accumulating multiple changes even over a few million years. At the same time, enhancers may remain functionally conserved, i.e. produce highly similar expression patterns, over long periods of time, even in the apparent absence of sequence conservation (Takahashi et al., 1999). Several enhancers are conserved between teleosts and mammals (e.g. Brenner et al., 2002). Furthermore, exchanges of Hox (Streit et al., 2002; Frasch et al., 1995; Pöpperl et al., 1995) and *Pax6*/*eyeless* (Xu et al., 1999) enhancer elements between flies, worms and vertebrates resulted in expression patterns that were interpreted as homologous. How universal are these results and what are the extent and the mechanisms of functional enhancer conservation in evolution?

To test the extent of conservation of cis-regulatory elements from distantly related organisms we generated transgenic *C. elegans* expressing the green fluorescent protein (GFP) under the control of tissue-specific enhancers from *D. melanogaster*. The nematode and arthropod lineages separated very early in animal evolution, prior to the ‘Cambrian explosion’ around 530 million years ago (Morris, 2000). Expression patterns of enhancer elements have been described in both species; furthermore, in the worm, expression pattern resolution is possible at the single-cell level. If functional conservation of enhancers is as prevalent as suggested by the many transcription factor genes that are functionally conserved across species, we would anticipate detecting expression of

GFP driven by a variety of *Drosophila* regulatory elements in homologous cell types in the worm.

Materials and methods

Enhancer fusion genes

Enhancer sequences from *Drosophila* were amplified from genomic DNA by PCR. We used Expand Long Template or Expand High Fidelity PCR Systems from Roche Molecular Biochemicals to decrease the number of sequence changes introduced during amplification. PCR products were then cloned upstream of the GFP gene into an appropriate cloning vector. We used pPD95.75 and pPD122.53 (the latter was modified to remove the nuclear localization signal) plasmids, both kind gifts from A. Fire, to generate translational and transcriptional fusions, respectively. Translational fusion genes contained enhancer elements, basal promoter, and the first several codons of the *Drosophila* gene fused to GFP. In transcriptional fusions, the enhancer element was the only segment of fly DNA placed upstream of the minimal *pes-10* promoter of *C. elegans*, which was not previously reported to produce an expression pattern alone. The identity of each construct was verified by restriction digestion and sequencing; DNA was prepared from multiple independent isolates and a mixture was used for injections. Identical procedures were used in preparing fusion genes containing putative enhancers from *C. briggsae*. Nucleotide sequences of enhancer elements, location of individual primers and the vectors used are given in Fig. S1 (at http://dev.biologists.org/supplemental).

Worm strains, injections and microscopy

Fusion genes were injected according to standard protocols (Mello et al., 1991) into either Bristol N2 or *pha-1* (*e2123*) animals. Enhancer-containing fusion genes were always injected at 50 ng/μl; whenever *pha-1* (*e2123*) worms were used, these were co-injected with a *pha-1* rescuing construct (Granato et al., 1994) at 2 ng/μl. Injection methods used for *C. briggsae* (AF16) were identical to those used for *C. elegans* N2. Multiple independent lines were examined for consistency of expression patterns. We established that fusion genes injected into *pha-1* (*e2123*) and N2 animals produce identical expression patterns. We noticed that animals from a number of transgenic lines displayed diffuse expression in the gut, primarily in the most anterior and posterior compartments, the PVT neuron, which has a projection as described by Aurelio et al. (Aurelio et al., 2002), not White et al. (White et al., 1986), and in several muscle cells of the pharynx (Fig. 1E). We observed this pattern of nonspecific expression for a number of fusion genes, including pPD95.75 and pPD122.53 vectors alone, in both the N2 and *pha-1* (*e2123*) genetic backgrounds. We therefore consider it to represent the 'background' pattern associated with the GFP transgene expression in the worm likely caused by the promiscuous transcriptional control in these tissues and/or a cryptic enhancer element within vector DNA. Occasionally this 'background' expression was also observed in vulva muscles and three rectal epithelial cells. All animals were initially evaluated under a dissecting microscope, and later examined in detail on a compound Zeiss Axioplan microscope; images were captured with the Open Lab software package and processed with Adobe Photoshop.

Results

Enhancer elements of *Drosophila* neuron-specific genes do not activate expression in the homologous *C. elegans* neurons

The most likely situation in which a cis-regulatory element would retain function when placed in a different species is in a genetic cascade where both the upstream transcription factor and the target genes are conserved across phylogeny. The pathway for generation of GABAergic neurons is one such example. The upstream specification pathway for many GABAergic neurons in nematodes is the homeobox transcription factor UNC-30, which is orthologous to Pitx genes in mammals as well as arthropods. Not only are these genes orthologous, but, in addition, expression of mouse *Pitx2* can rescue an *unc-30* mutant in *C. elegans* (Westmoreland et al., 2001). *unc-30* controls the differentiation of GABAergic neurons in nematodes, activating the expression of several target genes, including *unc-25* (glutamic acid decarboxylase) and *unc-47* (GABA transporter) genes (Eastman et al., 1999), both of which are also expressed in GABAergic neurons of arthropods and vertebrates. We generated and injected into *C. elegans* a construct containing a 4.5 kb fragment located immediately upstream of *Drosophila Ptx1* (an *unc-30* ortholog), which is a part of a larger (12 kb) enhancer element previously reported to direct *lacZ* expression in the endogenous pattern (Vorbuggen et al., 1997). We also tested large (8.5 and 6.5 kb) fragments upstream of *Gad1* (*unc-25*) and *CG8394.2* (*unc-47*), which we expected to contain enhancer activity. Neither *Drosophila Ptx1* nor *Drosophila CG8394.2* fusion genes activated expression above the background level, whereas *Drosophila Gad1::GFP* was abundantly expressed in glial cells of labial neurons and in several amphid neurons (Fig. 1A); none of these cells are GABAergic. Expression patterns of several other representative constructs are shown in Fig. 1; expression patterns of all constructs are summarized in Table 1. These data suggest that both transcriptional and translational fusion genes containing *Drosophila* enhancer elements are capable of directing expression in *C. elegans*, with neither type having a bias towards a cell type or tissue.

To broaden the search to other neural-specific enhancer elements, we tested constructs containing enhancers of genes expressed in different subsets of *Drosophila* nervous system: cholinergic neurons (acetylcholine esterase and choline acetyltransferase), catecholaminergic neurons (dopa decarboxylase) and FMRFergic neurons (FMRFamide). The 1.8 kb enhancer included in the *Drosophila ace::GFP* construct encompassed the sequence previously demonstrated to be sufficient for expression of a rescuing mini-gene (Hoffmann et al., 1992). Worms carrying this construct displayed strong pan-pharyngeal and vulva muscle expression as well as some background expression (gut and PVT), but we did not observe expression in the cholinergic neurons. Interestingly, *C. elegans* genome contains four *ace* genes, one of which, *ace-1*, is expressed in the pharynx, the body wall muscle and several head neurons (Combes et al., 2001), raising a possibility that the *C. elegans* transcription factor that activates *ace-1* expression in muscles can recognize enhancers of the *Drosophila ace* gene. It is unclear, however, whether the pharyngeal expression of *Drosophila ace::GFP* is due to specific recognition of conserved enhancer elements or to spurious activation, because we did not detect any expression in body muscles or head neurons.

The structure of *Cha* gene, including its enhancers and its close linkage with the acetylcholine transporter (*unc-17*), is highly conserved between worms, *Drosophila* and mammals, suggesting conservation of regulatory mechanisms (Rand and Nonet, 1997). Yet, faint expression of *Drosophila Cha::GFP* was only detected in several glial cells of labial neurons,

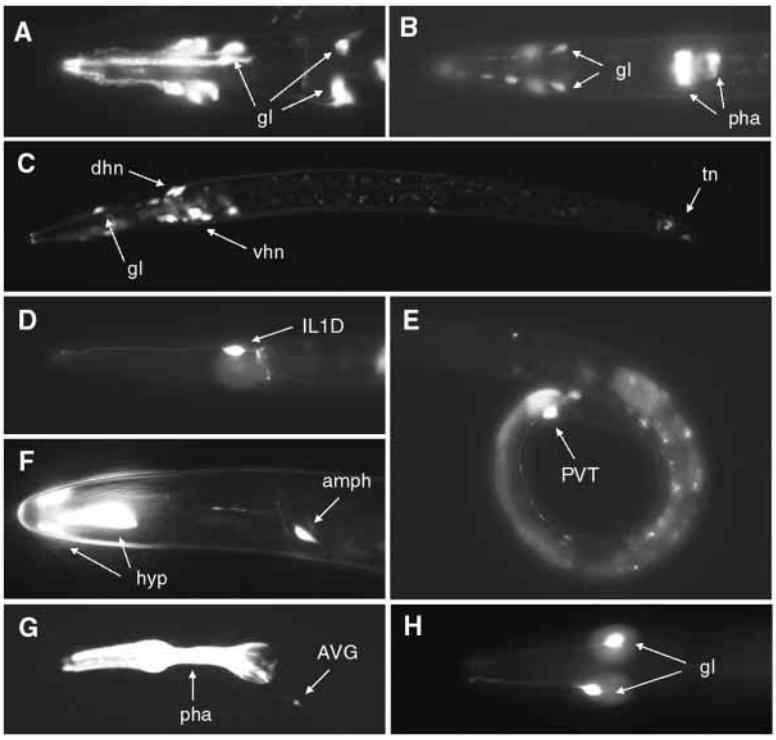

Fig. 1. Expression patterns of fusion genes containing tissue-specific enhancers of *Drosophila* in *C. elegans*. (A) *Drosophila Gad1::GFP* is expressed glial cells (gl) in the head. (B) *Drosophila Cha::GFP* is expressed in glial cells of labial neurons (gl) and several pharyngeal muscle cells (pha). (C) *Drosophila unc-119::GFP* is expressed in glial cells of labial neurons (gl), and several dorsal (dhn) and ventral (vhn) head neurons as well as in tail neurons (tn). (D) *Drosophila ey::GFP* is expressed in IL1D (L, R) and PVT. (E) *Drosophila eya::GFP* is expressed in PVT, in the gut and certain muscle cells of the pharynx. (F) *Drosophila nompA::GFP* is expressed in the head hypoderm (hyp) and in several amphid neurons (amph). (G) *Drosophila Mef2::GFP* is expressed throughout the pharynx (pha) and in a single interneuron AVG. (H) *Drosophila eve::GFP* is expressed in up to six glial (gl) cells of labial neurons.

several pharyngeal muscle cells and in the hypoderm (Fig. 1B), not in cholinergic neurons, whereas the same 3.3 kb fragment located immediately upstream of *Drosophila Cha* gene was shown to drive expression of *lacZ* in a subset of cholinergic neurons in the fly brain (Kitamoto et al., 1992).

Extensive analysis of transcriptional regulation of *Drosophila ddc* gene revealed that the cis-regulatory sequences required for endogenous gene expression in the nervous system are located within the 2.6 kb immediately upstream of the translation initiation site (Johnson et al., 1989). A *Drosophila ddc::GFP* construct containing this fragment was relatively strongly expressed in most pharyngeal muscle cells, a single amphid neuron, a single head interneuron (likely RICL or RIAL) and in PVT, but not in catecholaminergic neurons. Finally, *Drosophila FMRF::GFP* construct contained a 3.6 kb fragment upstream of the translation initiation site of *Drosophila* FMRFamide gene which was previously shown to be expressed in nearly all FMRFergic neurons in the fly (Benveniste and Taghert, 1999). We detected consistent expression of this fusion gene in most muscle cells in the pharynx, a single head interneuron (RMDDL, RMDL, RMF, or RMH) and three to five neurons in the ventral cord (DA or

Table 1. Expression patterns of constructs tested in this study

Construct*	Expected pattern	Observed pattern
Ptx1 (4.5 kb, C)	GABAergic neurons	Background
Gad1 (8.5 kb, C)	GABAergic neurons	Glial cells, amphid neurons
CG8394.2 (6.5 kb, L)	GABAergic neurons	Background
Ace (1.8 kb, C)	Cholinergic neurons	Pharyngeal and vulva muscles
Ddc (2.5 kb, L)	Serotonergic/dopaminergic neurons	Pharynx, one inter- and one amphid neuron
Cha (3.3 kb, L)	Cholinergic neurons	Glial cells of labial neurons, hypoderm
Fmrf (3.8 kb, L)	FMRFergic neurons	Pharynx, several VC neurons, one interneuron
Dm unc-119 (2.5 kb, L)	Pan-neuronal	Approximately 20 neurons, glia, pharynx, vulva
Dm ric-19 (2.2 kb, L)	Pan-neuronal	Pharynx
sng-1 (2.4 kb, L)	Pan-neuronal	Four to six glial cells in the head
ey (0.5 kb, C)	Sensory neurons	A pair of labial neurons
eya (0.3 kb, C)	Sensory neurons	Background
nompA (2 kb, L)	Glial cells of ciliated neurons	Head hypoderm, amphid neurons
nompC (1.6 kb, L)	Ciliated neurons	Background
Or23a (2.6 kb, L)	Olfactory (ciliated?) neurons	Background
Or46a (2.0 kb, L)	Olfactory (ciliated?) neurons	Background
Or47a (4 kb, L)	Olfactory (ciliated?) neurons	Background
Gr32d (3.8 kb, L)	Olfactory (ciliated?) neurons	Background
tin (0.9 kb, C)	Pharynx	Background
eve (1 kb, C)	Pharynx	Glial cells of labial neurons
tsh (1.2 kb, C)	Pharynx	Background, hypoderm
Mef2 (5.5 kb, C)	Pharynx	Pharynx, one interneuron

*The number in parenthesis is the size of the enhancer element used in the construct. C, transcriptional fusion; L, translational fusion.

VA). Again, none of these detected expression patterns could be considered homologous to the endogenous patterns in the fly.

In *C. elegans*, a gene *unc-119* is expressed throughout the nervous system (Maduro and Pilgrim, 1995). Its ortholog in *Drosophila* is also expressed in essentially all neurons and is functionally conserved (Maduro et al., 2000). We therefore tested the expression pattern in *C. elegans* of a GFP fusion gene containing 2.5 kb upstream of the *Drosophila* gene, which, although not previously tested in flies, was expected to contain at least some of the regulatory elements. As shown in Fig. 1C, strong expression can be seen in up to 10 neurons in the head, four to six in the tail, and several in the body (including HSN and SDQs). Although not all neurons expressed GFP, and additional expression was seen in glial cells, the pharynx and the vulva, it may be significant that the nervous system was the predominant site of expression. Our studies of the 5′ regulatory region of the *C. elegans unc-119* suggest that the pan-neural expression pattern of this gene is assembled in a 'piecemeal' fashion, probably mediated by the action of independent cis-regulatory elements (I.R. and G.R., unpublished). It is plausible that the *Drosophila unc-119* gene is similarly regulated and that some of its enhancer elements are recognized by the same transcription factors that regulate expression of *C. elegans unc-119* gene. Encouraged by this observation, we tested upstream sequences (2.2 and 2.4 kb) of *Drosophila* orthologs of two additional *C. elegans* pan-neural genes, *Drosophila ric-19* and *sng-1* (synaptogyrin), which are expected to be expressed throughout the nervous system (Pilon et al., 2000; Zhao and Nonet, 2001). The former was faintly expressed in the pharynx, whereas the latter was restricted to four to six glial cells in the head; neither therefore was expressed pan-neurally. Thus, *unc-119* is unique in the conservation of its neuronal regulation.

Although *C. elegans* displays light sensing behaviors (Burr, 1985), it lacks morphologically defined eyes. Because a key transcription factor in the eye specification program is highly conserved among all animals – *eyeless* in *Drosophila*, *Pax6* in vertebrates and *vab-3* in *C. elegans* (Carroll et al., 2001; Davidson, 2001) – we also tested enhancers of *Drosophila* eye-specific genes in *C. elegans*. In addition, an evolutionary connection has been proposed to exist between thermosensory neurons of nematodes and photoreceptors of other animals (Satterlee et al., 2001; Svendsen and McGhee, 1995). We generated fusion genes containing enhancer elements of *eyeless* (0.5 kb) and *eyes absent* (0.3 kb) genes; both of these sequences were previously shown to direct reporter gene expression in eye primordia in *Drosophila* (Zimmerman et al., 2000; Hauck et al., 1999). Worms carrying *Drosophila ey::GFP* transgene showed strong and consistent expression in a pair of labial neurons in the head, IL1D (L, R), and the PVT neuron (Fig. 1D), cells that probably do not express *vab-3* (A. Chisholm, personal communication), although it is tantalizing that IL1D (L, R) are a pair of anterior sensory neurons. *Drosophila eya::GFP*-carrying worms showed no expression in any head neurons (Fig. 1E). Therefore, two enhancers that are expressed in the same cells in the fly are not co-expressed in the worm.

Next, we tested fusion genes containing putative enhancers of *nompC* (Walker et al., 2000) and *nompA* (Chung et al., 2001), which in *Drosophila* are expressed by ciliated sensory neurons and their glial support cells, respectively. As the endogenous enhancer of *Drosophila nompC* was not characterized in detail, our construct included a 1.7 kb fragment immediately upstream of the translation initiation site, covering the interval between *nompC* and the upstream gene. This fusion gene was not expressed above the background level in *C. elegans*. The putative enhancer (2 kb) included in the *Drosophila nompA* fusion gene also extended between the site of translation initiation and the upstream gene and covered the sequence capable of directing GFP expression in the endogenous pattern (Chung et al., 2001), although coding sequences and downstream introns were absent from our construct. We detected strong GFP expression in six to eight amphid neurons and several cells of anterior hypoderm (Fig. 1F). Neither pattern could be considered homologous to the endogenous expression domain in the fly, nor, in the case of *nompC* construct, to the pattern of the putative worm ortholog (Walker et al., 2000).

Finally, we tested enhancers of four olfactory/gustatory receptors expressed in sensory neurons in *Drosophila* (Scott et al., 2001; Gao et al., 2000; Vosshall et al., 2000). These constructs, *or23a* (2.6 kb), *or46a* (2.0 kb), *or47a* (4 kb) and *gr32d* (3.8 kb), contained sequences immediately upstream of receptor genes and were previously demonstrated to be sufficient to drive reporter gene expression in the endogenous pattern. We therefore expected them to be expressed in ciliated sensory neurons, where worm olfactory receptors are expressed. However, we observed no expression of these fusion genes in *C. elegans*.

Expression of *Drosophila* heart-specific enhancers is not confined to *C. elegans* pharynx

To test whether enhancers of genes expressed in tissues other than neurons are conserved between worms and flies, we examined expression patterns of fusion genes containing *Drosophila* heart-specific enhancers. Although nematodes do not have a heart, there are some functional similarities between the nematode pharynx and the vertebrate and the insect heart (Okkema et al., 1997). Aspects of heart patterning are highly conserved in evolution (Fishman and Olson, 1997), including members of the *tinman* family of transcription factors, which are functionally interchangeable between worms and vertebrates (Haun et al., 1998).

We generated fusion genes containing entire heart-specific enhancers of *Drosophila tinman* (0.9 kb) (Yin et al., 1997), *even-skipped* (1 kb) (Halfon et al., 2000), *teashirt* (1.2 kb) (McCormick et al., 1995) and *Mef2* (5.5 kb) (Cripps et al., 1999) genes. All four of these enhancers have been previously demonstrated to direct expression of reporter genes in the *Drosophila* heart. Because all four genes are involved in a conserved pathway of cardiomyocyte differentiation, we expected that the constructs would be expressed in the pharynx. *Drosophila Mef2::GFP* was strongly expressed throughout the pharynx and in a single interneuron – AVG (Fig. 1G). Two other fusion genes, *Drosophila tin::GFP* and *Drosophila tsh::GFP*, were expressed in the 'background' pattern and in seam cells, whereas *Drosophila eve::GFP* was consistently and strongly expressed in up to six glial cells of labial neurons (Fig. 1H). Therefore, one of four heart-specific enhancers, *Drosophila Mef2*, displayed an expression pattern consistent with the conservation of transcriptional control between insects and nematodes. It is possible that this enhancer element is

functionally conserved between worms and flies, although it is also possible that this instance represents a convergently acquired similarity of expression patterns.

Orthologous enhancers from *C. briggsae* and *C. elegans* produce similar, yet distinct, expression patterns

Because many *Drosophila* enhancers showed little or no conservation of tissue-specific expression in *C. elegans*, we assessed the functional conservation of enhancer elements between more closely related species. We compared expression patterns driven by orthologous enhancers from *C. elegans* and *C. briggsae*, two nematode species that retain nearly identical morphology (Fitch and Thomas, 1997), but are estimated to have diverged about 50-120 million years ago (Coghlan and Wolfe, 2002), or about 10 times more recently than arthropods and nematodes. We chose enhancers of two genes, *unc-25* and *unc-47*, because they are relatively short and well characterized. As shown in Fig. 3A, in *C. elegans* both genes are expressed exclusively in the 26 GABAergic neurons – four RMEs, AVL, RIS, six DDs, 13 VDs and DBA (McIntire et al., 1993).

We generated fusion genes containing 930 and 835 nucleotides upstream of the ATG codons of *C. briggsae unc-25* and *unc-47* genes, respectively; orthologous fragments in *C. elegans* are sufficient to direct expression in the endogenous pattern (Eastman et al., 1999). Alignments of cognate enhancer pairs (Fig. 2) revealed that sequence conservation is distributed unevenly – blocks of nearly identical sequence are interspersed with gaps or 'spacers' of variable length. This trend is particularly pronounced in the 200-300 nucleotides immediately adjacent to the ATG, whereas changes are more evenly distributed in the more upstream regions.

Expression patterns generated by the *C. briggsae unc-25* enhancer (*cb unc-25*) were qualitatively similar in *C. elegans* and *C. briggsae*. We observed expression in most DD and VD neurons, less often in the RMEs, AVL and RIS and never in DVB. Therefore these expression patterns were very similar to that of *unc-25* in *C. elegans* (*ce unc-25*) (Eastman et al., 1999) (Y. Jin, personal communication). We did notice however that the heterospecific enhancer/host combination (*cb unc-25* in *C. elegans*) resulted in weaker expression (also true for *unc-47* enhancers), which was more mosaic with respect to the cells expressing GFP. This result was confirmed in multiple, independently derived lines and was previously reported in studies of hetero- versus homospecific enhancer/host combinations (Molin et al., 2000; Ludwig et al., 1998).

In contrast to the *unc-25* enhancer, both *ce unc-47::GFP* and *cb unc-47::GFP* were expressed in all 26 GABAergic neurons of both *C. elegans* and *C. briggsae*. Additionally, *cb unc-47::GFP* was strongly expressed in SDQ (L, R) in *C. elegans* and weakly in SDQL in *C. briggsae* (Fig. 3). The two SDQ neurons are descendants of the Q (L, R) blast cells and are not GABAergic (Rand and Nonet, 1997; McIntire et al., 1993; Guastella et al., 1991). We sought to identify the cis-element(s) within the *cb unc-47* enhancer responsible for SDQ (L, R) expression. We generated two enhancer fusion genes – one encompassed the most proximal 250 nucleotides containing several highly conserved sequences upstream the ATG and the other the remaining 580 nucleotides (Fig. 4). When these were introduced in *C. elegans*, the former recapitulated almost the entire pattern of the original *cb unc-47* enhancer, with the exception of RME (D, V), which either did not express GFP or were very faint; SDQ (L, R) expression was also conspicuously absent. We tested the distal 580 nucleotide fragment alone, in direct and reverse orientation and as two tandemly repeated copies in direct orientation. Expression patterns of these three fusion genes were similar, in two GABAergic neurons: RME (D, V), as well as in two pairs of amphid neurons, in one pharyngeal neuron and the 'background' pattern (PVT and in the gut). We observed no expression in SDQ neurons. These results therefore suggest that the novel expression pattern characteristic of the *cb unc-47* enhancer probably resulted from a synergistic interaction between the elements within the distal and the proximal enhancer fragments, or less likely by an element at the –250 site.

Discussion

Tissue-specificity of enhancers is often not conserved between insects and nematodes

Our results (Fig. 1, Table 1) suggest that enhancer elements from a distantly related lineage often are not properly recognized in *C. elegans*, because few of *Drosophila* enhancers were expressed in homologous patterns in the worm. Observed similarities (*Drosophila Mef2* expression in the pharynx and *Drosophila unc-119* in the neurons) may be a consequence of stringent selection acting upon these enhancers, particularly if they contain relatively few individual binding sites. Alternatively, it may be a reflection of serendipitous occurrence of binding sites recognized in particular tissues in *C. elegans*. Although a number of previous studies reported functional conservation of enhancers from distantly related species, most of those were *Hox* gene enhancers (Streit et al., 2002; Frasch et al., 1995; Pöpperl et al., 1995). Extending these observations to other genes may be confounded by the peculiar mode of regulation inherent to the Hox family, with autoregulation and conservation of gene order within paralogous clusters which possibly constrains cis-regulatory evolution. Additionally, most comparisons also involved less distantly related species pairs, e.g. mammal-bony fish (Brenner et al., 2002). At least in some instances when the evolutionary distances between compared species were sufficiently large, little or no functional conservation was observed (Jones et al., 2002; Locascio et al., 1999). It is therefore likely that arthropods and nematodes are separated by an evolutionary distance over which little functional conservation is retained by the majority of enhancers.

Conservation and divergence between enhancers of *C. elegans* and *C. briggsae*

The results of our tests of functional conservation of *unc-25* and *unc-47* enhancers between *C. elegans* and *C. briggsae* suggest that despite divergence of primary sequence and substantial changes in spacing between conserved blocks of sequence, these two sets of enhancers largely maintained their function over 50-120 million years separating the two species. We noticed that in the case of both *unc-25* and *unc-47*, expression was stronger and more consistent in the homospecific enhancer/host species combination, similar to what was seen in other enhancer comparisons (Molin et al.,

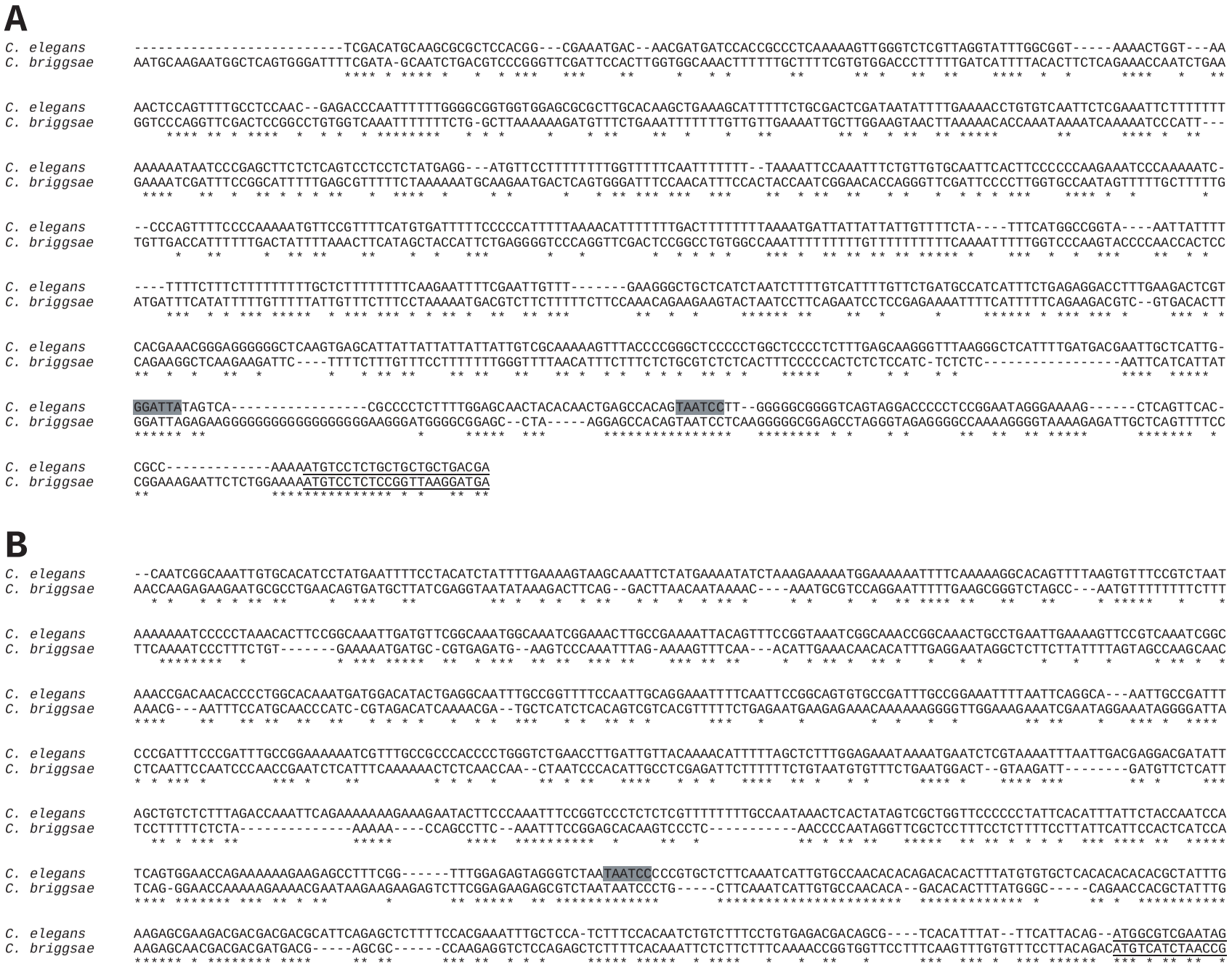

Fig. 2. Alignments of upstream regulatory sequences of (A) *unc-25* and (B) *unc-47* of *C. elegans* and *C. briggsae*. –, alignment gaps; *, identical nucleotides. *unc-30*-binding sites are gray, as shown previously (Eastman et al., 1999). Translated sequences are underlined.

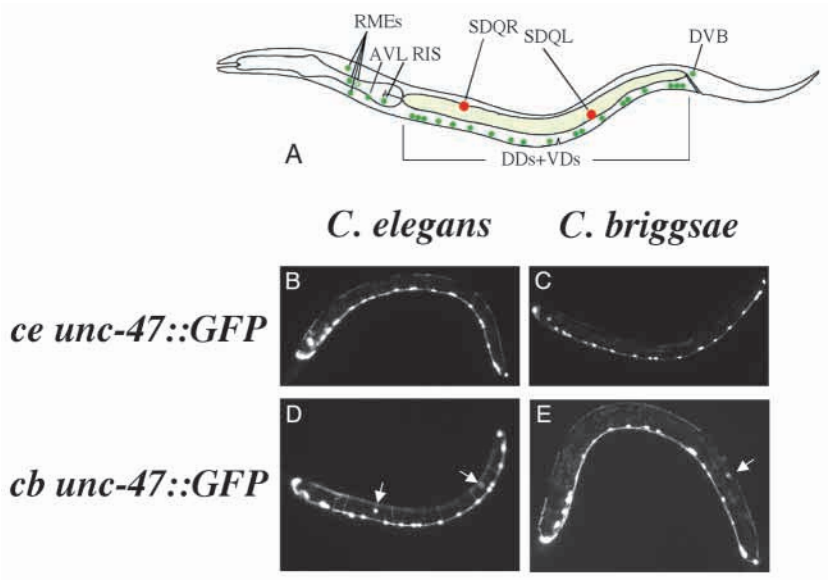

Fig. 3. Functional comparisons of *unc-47* enhancers between *C. elegans* and *C. briggsae*. (A) Schematic representation of expression patterns. 26 GABAergic neurons – four RMEs, AVL, RIS, six DDs, 13 VDs and DVB – are shown in green. SDQ (L, R) are shown in red. (B-E) Expression patterns of *ce unc-47::GFP* (B,C) and *cb unc-47::GFP* (D,E) in both *C. elegans* (B,D) and *C. briggsae* (C,E). Note that in all four panels most, if not all, of the 26 GABAergic cells express GFP. Arrows indicate SDQ (L, R) in D and SDQL in E.

2000; Ludwig et al., 1998). It is of interest that structurally similar (Fig. 2), orthologous fragments of *unc-47* enhancer from *C. elegans* and *C. briggsae* are functionally nonequivalent (Fig. 3). Specifically, expression in SDQ (L, R) is a property inherent to the *cb unc-47*, but not *ce unc-47*, enhancer. Our results further suggest that a synergistic interaction between the distal and the proximal enhancer fragments, rather than the acquisition of a specific site, results in the SDQ (L, R) expression pattern.

Recently, Romano and Wray (Romano and Wray, 2003) examined functional conservation of enhancer elements between two species of sea urchins which are separated by approximately the same genetic distance as *C. briggsae* and *C. elegans*. Although overall expression patterns observed in heterologous enhancer/host tests were similar for these two species, there were also notable differences, including ectopic expression. Remarkable similarity between these results and our observations (Fig. 3), further supports the notion of rapid evolution of transcription factors and cis-regulatory elements and suggests that examination of orthologous enhancers from intermediately divergent species will likely shed light on molecular bases of evolutionary change.

Our functional analysis of *cb unc-47* enhancer provides evidence that expression of this gene is regulated by genetically distinct mechanisms in RME (D, V) versus RME (L, R) cells, because proximal enhancer was predominantly expressed in the left/right pair, whereas the distal enhancer only in the dorsal/ventral pair (Fig. 4). Interestingly, in *C. elegans*, expression of *lim-6*, a gene possibly acting in specification of non-D GABA cells, is detected in the L/R, not the D/V pair (Hobert et al., 1999), further indicating that these pairs are genetically distinct. Similarly, in *C. elegans* expression of *unc-47* may be regulated by different mechanisms in RME (L, R) and RME (D, V) cells (Y. Jin, personal communication).

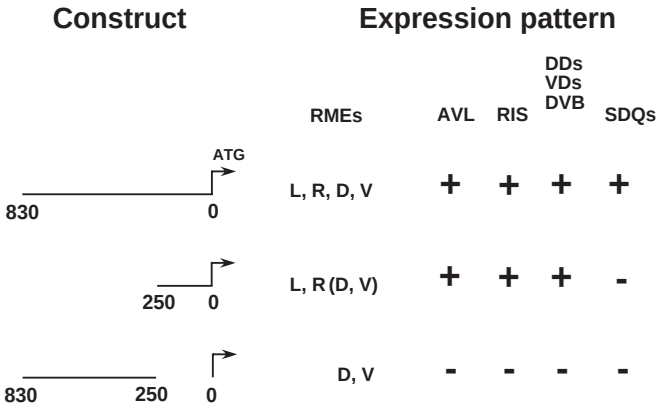

Fig. 4. Functional dissection of *cb unc-47* enhancer to identify element(s) responsible for expression in SDQ (L, R). +, strong and consistent expression in particular cells; -, complete lack of expression. (D, V) indicates weak and inconsistent expression of the proximal enhancer in RME (D, V) cells. Note that SDQ (L, R) are the only cells, expression in which is not activated by either of the two shorter enhancer fusion genes.

Co-evolution of enhancers and transcription factors maintains homologous patterns of gene expression

Sequence comparisons of *unc-25* and *unc-47* enhancers between *C. elegans* and *C. briggsae* (Fig. 2) suggest that both the relative spacing of conserved blocks and the sequences within such blocks, diverge relatively rapidly. Similar patterns of sequence variation were previously observed in enhancer comparisons of drosophilids (Ludwig et al., 1998) and rhabditid nematodes (Webb et al., 2002). Apparently, during the initial stages of species divergence there is a large degree of functional conservation that persists despite the accumulation of a considerable number of differences within enhancers. In some instances, functional equivalence is maintained even between highly divergent regulatory elements with distinct internal organization (Takahashi et al., 1999).

The co-evolution between transcription factors and their binding sites is the most plausible hypothesis to account for these observations. According to this model, individual binding sites within enhancer elements arise and vanish on the time scale of a few million years. If one site disappears while

another arises at a different location within the enhancer, there will be an appearance of 'reshuffling' of binding sites. To counterbalance this constant change, transcription factors co-evolve with their binding targets (Shaw et al., 2002). Because concerted action of a number of proteins is required for transcriptional initiation (Tjian and Maniatis, 1994), perhaps the most important aspect of this co-evolution is not the adjustment of binding affinity to a newly evolved site, but the changes in protein-protein interactions with other transcription factors whose binding sites are located nearby. Recent studies revealed that while retaining their overall functions, transcription factors can evolve novel roles by acquiring amino acid replacements in their protein-protein interaction domains (Hsia and McGinnis, 2003; Galant and Carroll, 2002; Ronshaugen et al., 2002). It is known that not only orthologous transcription factors, but even more distantly related family members, often recognize similar DNA sequences (Conlon et al., 2001). It is also well established that DNA-binding domains of transcription factors evolve considerably slower than the domains involved in protein-protein interactions (it is true for transcription factors in this study, see Figs S2 and S3 at http://dev.biologists.org/supplemental/). Moreover, changes in DNA-binding specificity would affect multiple target genes, whereas because of the modular nature of transcription factors, interactions with one partner may be adjusted without compromising other functions. Over time, 'reshuffling' of individual binding sites gives an appearance of considerable sequence divergence, yet the complex of transcription factors that assembles on an enhancer may be largely the same, resulting in the conservation of gene expression patterns.

The co-evolution model can be used to explain a seemingly paradoxical observation: individual transcription factors are often functionally conserved over very large phylogenetic distances (Grens et al., 1995), whereas our results suggest that enhancer sequences from an arthropod often are not properly recognized in a nematode. If we consider two distantly related species A and B, enhancers of orthologous target genes would have little detectable sequence similarity due to multiple rounds of 'reshuffling', yet two sets of orthologous transcription factors may regulate their expression, each optimally co-evolved to recognize its target (Fig. 5A,B). When placed into species A, which is mutant for a particular transcription factor, an ortholog from species B could bind to an appropriate target site (Fig. 5C). Although this binding may be weaker than to its native target and its interaction with other transcription factors assembled on the enhancer may be less specific than to its native binding partners; in the framework of an experiment it may still rescue the mutation because some binding and some interaction are retained. If, however, an enhancer from species A is placed into species B (Fig. 5D), it is unlikely to be expressed because none of the interactions between transcription factors required for transcriptional activation are likely occur. Therefore, co-evolution of rapidly diverging enhancer elements with their transcription factors may be one of the molecular mechanisms underlying a commonly observed phenomenon of 'developmental systems drift' (True and Haag, 2001), in which apparently homologous traits in distantly related species are determined by distinct genetic programs.

Conclusions

We presented evidence that although several enhancers of *Drosophila melanogaster* have retained their tissue-specific functions, most are not appropriately recognized in *C. elegans*. However, orthologous enhancers from two nematode species, *C. elegans* and *C. briggsae*, are largely functionally conserved, despite considerable sequence divergence. As we identified specific functional differences between *C. elegans* and *C. briggsae* enhancers, it is likely that comparisons of orthologous enhancers from species pairs that diverged 10-100 million years ago will uncover instances of functional divergence caused by a relatively small number of nucleotide differences. Such studies would contribute to our understanding of functional evolution of regulatory elements and molecular bases of morphological evolution. Finally, it is likely that enhancers that evolve relatively rapidly co-evolve with their binding factors; it is their cohesive interaction, not the primary structure of enhancer elements, that is preserved by selection over long periods of time and results in the conservation of gene expression patterns between distantly related species.

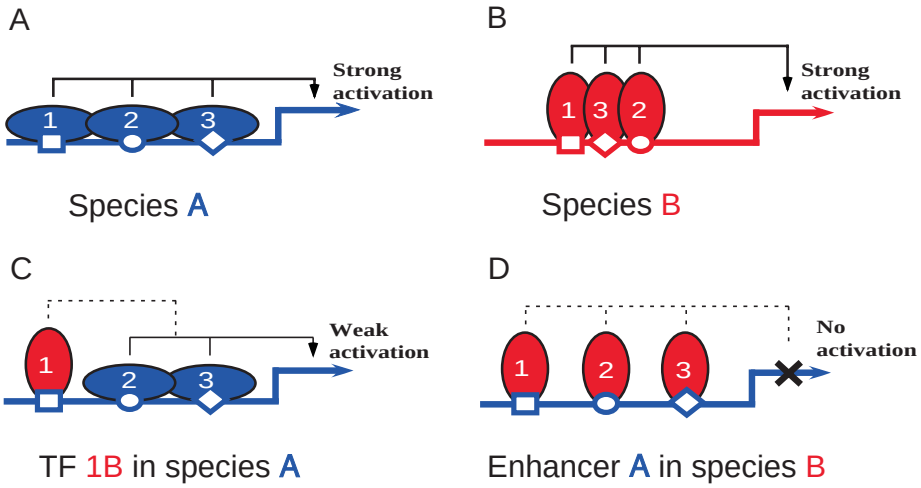

Fig. 5. Consequences of enhancer-transcription factor co-evolution. (A,B) Sets of orthologous transcription factors control expression of an orthologous target in species A (blue) and B (red). Note that although the order of individual binding sites is rearranged, in both cases transcription factors are co-adapted, as reflected by their different shapes, to form a complex and result in strong activation of expression. (C) If transcription factor 1 in species A is replaced by its ortholog from species B, it could bind to the target previously occupied by its ortholog. It could also interact although less well (as indicated with a broken line) with other transcription factors bound to the enhancer, resulting in weaker transcriptional activation. (D) If an entire enhancer is placed into a heterospecific context, individual transcription factors may be able to bind to their respective target sequences. Their interactions, however, are likely to be greatly hampered, thus resulting in no transcriptional activation or in activation in a different pattern because of serendipitous occurrence of binding sites recognized in other tissues.

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