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Conserved sequence block clustering and flanking inter-cluster flexibility delineate enhancers that regulate *nerfin-1* expression during *Drosophila* CNS development

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Abstract

We have identified clusters of conserved sequences constituting discrete modular enhancers within the *Drosophila nerfin-1* locus. *nerfin-1* encodes a Zn-finger transcription factor that directs pioneer interneuron axon guidance. *nerfin-1* mRNA is detected in many early delaminating neuroblasts, ganglion mother cells and transiently in nascent neurons. The comparative genomics analysis program *EvoPrinter* revealed conserved sequence blocks both upstream and downstream of the transcribed region. By using the aligning regions of different drosophilids as the reference DNA, *EvoPrinter* detects sequence length flexibility between clusters of conserved sequences and thus facilitates differentiation between closely associated modular enhancers. Expression analysis of enhancer-reporter transgenes identified enhancers that drive expression in different regions of the developing embryonic and adult nervous system, including subsets of embryonic CNS neuroblasts, GMCs, neurons and PNS neurons. In summary, *EvoPrinter* facilitates the discovery and analysis of enhancers that control crucial aspects of *nerfin-1* expression.

Keywords

Enhancers; Drosophila; Nervous system development; EvoPrinter; phylogenetic footprinting; Nerfin-1; Transcription factor; Embryonic expression; Larval expression

The *Drosophila nerfin-1* gene encodes a transcription factor (TF) that is essential for the wild-type expression of a subset of axon guidance genes in nascent neurons (Kuzin et al., 2005). The Nerfin-1 protein belongs to a highly conserved subfamily of transcriptional regulators, which are identified by a unique set of tandem Zn-fingers known as the EIN-domain (Stivers et al., 2000). Nerfin-1 orthologs function in all metazoans, e.g., nematodes (Desai and Horvitz, 1989) and mammals (Goto et al., 1992), where they are expressed in the developing nervous system (Wu et al., 2001; Breslin et al., 2003). *Drosophila nerfin-1* mRNA expression is detected in many early delaminating CNS neuroblasts (NBs), ganglion mother cells (GMCs) and nascent neurons (Stivers et al., 2000; Kuzin et al., 2005). Temporal regulation of *nerfin-1* expression appears to be functionally critical, based on the observation

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that prolonged ectopic expression of Nerfin-1 protein in neurons is lethal (Kuzin et al., 2005).

Work with mammals (Visel et al., 2007) and *Drosophila* (Berman et al., 2004; Papatsenko et al., 2006) indicates that enhancers contain clusters of TF DNA-binding sites: cross-species comparisons reveal that many of these sites are highly conserved. Use of the phylogenetic footprinting tool *EvoPrinter* has revealed that mammalian and *Drosophila* enhancers contain clusters of highly conserved sequences blocks (CSBs) (Odenwald et al., 2005; Brody et al., 2007). For example, the mammalian Dll1 regulatory region consists of four enhancers, each made up of a cluster of CSBs: two of these drive Dll1 expression in the CNS and two in mesoderm (Beckers et al., 1999; Brody et al., 2007). Likewise the *Drosophila Kruppel* segmentation (Hoch et al., 1990,1991,1992) and *snail* neural and mesodermal enhancers (Ip et al., 1994) all consist of clusters of CSBs flanked by less-conserved DNA (Odenwald et al., 2005; Brody et al., 2007). Functional analysis of enhancers reveals that they contain conserved sites that bind both positive and negative regulators of enhancer activity (Barolo and Posakony, 2002; Liu et al., 2008).

Reported here is the identification of the multiple enhancers that control different aspects of the *nerfin-1* gene expression pattern in the developing nervous system. Our comparative genomics analysis reveals that the *nerfin-1* transcribed sequence is flanked by multiple clusters of CSBs that are separated by less-conserved DNA. Analysis of Enhancer-reporter transgenes reveals that each of the different CSB clusters functions as a discrete enhancer that regulates different aspects of *nerfin-1* expression. For example, the NB, eye disc, and neuron enhancers are each made up of CSB clusters. *EvoPrinter* analysis also revealed that the less-conserved DNA between CSB clusters displays greater cross-species sequence length variability when compared to the sequences within enhancers, and reporter transgene analysis reveals that these less-conserved sequences lack enhancer activity. We conclude that the identification of both CSB clusters and sequence length variability between conserved clusters provides a basis for the discovery of modular *cis*-regulatory DNAs that control different spatial/temporal phases of dynamic gene expression.

1. Results and discussion

1.1. Comparative genomic analysis of nerfin-1 and flanking sequences

Our prior studies have shown that an 11 kb genomic DNA fragment that includes the *nerfin-1* transcribed sequence and 5780 bp of 5′ and 2130 bp of 3′ flanking sequence can rescue the *nerfin-1*^{null} lethal phenotype and restore its wild-type expression pattern (Kuzin et al., 2005). *EvoPrinter* analysis of the *nerfin-1* rescue fragment reveals four regions of DNA sequence conservation: flanking the transcribed sequence, within the open reading frame and within both the 5′ and 3′ UTRs (Fig. 1). Within the *nerfin-1* open reading frame, the conserved bases encode the highly conserved DNA-binding domain (Stivers et al., 2000) and the conserved bases within the 3′UTR correspond to multiple micro-RNA binding sites (Kuzin et al., 2007). These conserved micro-RNA binding sites regulate *nerfin-1* spatial and temporal translation dynamics in the developing nervous system. The 5′ upstream region of the *nerfin-1* rescue fragment contains 77 CSBs with an average sequence length of 17.5 bp – slightly higher than the average CSB length reported previously, 13 bp, for a larger selection

of enhancers (Brody et al., 2007). The longest CSB among these conserved clusters was 42 bases in length.

In this study of *nerfin-1* regulation we have used the distribution of the CSBs flanking the *nerfin-1* structural gene to formulate a strategy for the functional dissection of *cis*-regulatory sequences. Our analysis is based on the idea that enhancers consist of clusters of conserved bases (see for example Woolfe et al., 2005; Sosinsky et al., 2007). The clustering of CSBs within the non-transcribed regions of the *nerfin-1* locus indicates that multiple enhancers regulate *nerfin-1* expression. We noticed that the length of the less-conserved sequences between the CSB clusters varies between species. The variability in sequence length between and within clusters is demonstrated in Fig. 2, which shows the mean cluster size and coefficient of variation for inter- and intra-cluster sequences. In each case the variability of the inter-cluster non-conserved regions was greater than that of less-conserved regions between CSBs within clusters. The degree of sequence length variability in the between cluster regions was most pronounced when evolutionarily distant species were compared. For example, the sequence length variability between clusters 5 and 6 in *Drosophila melanogaster* and *Drosophila mojavensis* is considerably greater than the sequence length variability within each of the two clusters (Fig. 2B and C). As a notable exception to the higher inter-cluster variation, the intra-cluster region within fragment #6 shows a coefficient of variation between the low intra-cluster variation of other 5′ clusters and the higher inter-cluster variations. The greater intra-cluster variation in fragment #6 can be explained by the fact that it contains multiple enhancers that can act independently of each other (see below).

1.2. nerfin-1 expression is regulated by multiple discrete enhancers

To determine whether the CSB clusters detected by *EvoPrinter* function as independent enhancers, we generated enhancer-reporter transgenes of the different regions detected in the comparative genomics analysis. Fragments analyzed for enhancer activity in reporter studies are depicted in Fig. 1, with the results of the reporter transgene expression analysis summarized in Table 1 (see Supplemental data Table 1 for the exact fragment boundaries). For each construct, multiple independent homozygous transgenic lines were tested to ensure that they had the same spatial and temporal transgene expression dynamics. We attribute the uniform expression dynamics observed in independent transformant lines to the presence of gypsy insulators flanking the enhancer-reporter transgenes within the pRed H-Stinger vector (Barolo et al., 2004).

The genomic region consisting of all the upstream CSB clusters (fragment #1) drives reporter DsRed expression in CNS NBs, GMCs and neurons and in PNS neurons (Fig. 3A and data not shown), recapitulating most aspects of the embryonic mRNA and protein expression pattern of *nerfin-1* (Stivers et al., 2000 and Kuzin et al., 2005). Reporter expression in CNS NBs matches that of endogenous mRNA, which is expressed in most early NBs, but it does not reflect the post-transcriptional regulation by micro-RNAs, restricting mRNA expression (Kuzin et al., 2007). Likewise reporter expression in PNS neurons (Kuzin et al., 2005) is not downregulated as fast as the endogenous *nerfin-1* message, since it does not reflect the restriction of PNS mRNA expression by micro-RNAs.

The full length fragment also activates reporter expression in the 3rd instar larva eye disc, CNS NBs and nascent neurons (data not shown).

Analysis of individual CSB clusters within fragment #1 revealed that the overall pattern observed was a result of the action of multiple enhancers. Genomic regions #2 and #3, which span adjacent CSB clusters, activated reporter expression in different subsets of embryonic ventral cord neurons (Fig. 3B and C, respectively). Genomic region #4 activated expression in a wider subset of neurons during embryonic stage 15 (Fig. 3D). No larval expression was detected for transgenes #2, #3 and #4. Genomic region #5 did not activate expression during embryonic development but drove reporter expression in a ring of cells in the optic lobe of 3rd instar larva (Fig. 3E). Expression in the larval optic lobe appears to coincide with the outer proliferative center of the optic lobe (Egger et al., 2007; Colonques et al., 2007). These cells are likely to be newly formed neural precursors whose progeny generate the outer medulla and lamina neurons and the glia of the optic lobe. Fragment #6, which contains multiple clusters of CSBs, activated expression in a subset of early NBs as well as in GMC and PNS neurons (Fig. 3F and G and data not shown).

Testing the 3′ region of the *nerfin-1* locus for enhancer activity, we found that the conserved sequences contained in fragment #8 activated reporter expression in a subset of lateral ventral cord NBs during embryonic stage 12 and in a subset of cells in the developing optic lobe of the 3rd instar larva (Fig. 3H and I). The longer fragments (#7 and #13) gave the same expression pattern as fragment #8. Previous studies have revealed that the conserved sequences in the 3′ UTR correspond to micro-RNA binding sites that are involved in the post-transcriptional regulation of the *nerfin-1* transcript (Kuzin et al., 2007). Genomic regions #9 and #10 that lack CSBs failed to demonstrate any enhancer activity in embryos or larvae (data not shown).

Our initial round of enhancer-reporter constructs, described above, revealed that the early NB enhancer resided within the multi-cluster fragment #6. To further define the NB enhancer, we undertook the truncation analysis of fragment #6. Our previous studies suggested independent regulation of *nerfin-1* in ventral cord NBs and GMCs since the temporal course of NB expression differed markedly from that of ventral cord GMC expression (Stivers et al., 2000). In an attempt to localize the *nerfin-1* NB enhancer and distinguish it from the GMC enhancer, we designed two additional constructs (#11 and #12) that contained respectively the conserved part of the upper region and the lower region of fragment #6. Fragment #11 revealed no detectable embryonic expression (data not shown; and see below). Fragment #12 was sufficient for combined GMC, PNS and neuronal expression (Fig. 3J and K), and also drove expression in neurons behind the morphogenetic furrow of the eye imaginal disc (Fig. 3L). Extending fragment #12 distally to include the conserved putative bHLH DNA-binding E-box sequences (CAGCTG) did not expand its expression to NBs (data not shown). This analysis indicates that fragment #6 contains at least two distinct enhancer activities: this region drives reporter expression in many early NBs and GMCs, and the proximal sequences of fragment #6 activates expression in GMCs and neurons and in photoreceptor neurons of the eye imaginal disc. Further truncation analysis of fragment 6 revealed that the *nerfin-1* early NB enhancer was located 249 bp upstream of the predicted transcription start site and spans 567 bp (Fig. 4).

The early NB enhancer drives expression during a narrow temporal window of ~2 h, with expression in many but not all S1 and S2 NBs starting at late stage 8 (Fig. 5A). Expression was noticeably absent from row 1 NBs (see arrows in Fig. 5A). A comparison of transgene and endogenous mRNA expression patterns revealed no significant differences onset, extent or down-regulation of expression (compare Figs. 5 and 6A). By stage 10, expression is detected in many cephalic lobe NBs and was significantly downregulated in the ventral cord NBs. By stage 11, expression was absent from the ventral cord and remained in only a few brain NBs. In general, detection of the reporter mRNA levels confirmed the expression pattern detected by immunostaining (data not shown), however, the reporter protein perdures much longer than its transcript.

It is noteworthy that, with the exception of fragment #11, each of the different *nerfin-1* enhancers identified contains conserved bHLH TF DNA-binding sites, which may be targeted by proneural TFs (reviewed by Ledent and Vervoort, 2001, Gibert and Simpson, 2003). Of special interest are the three evolutionarily conserved CAGCTG sites within the early NB enhancer, highlighted in its *EvoPrint* (Fig. 5B) – identical sites are also present in multiple copies in the *deadpan* NB enhancer (Emery and Bier, 1995; Brody et al., 2008) and in the nervy NB enhancer (Brody et al., 2008), suggesting that there is specific proneural input into these enhancers. Previous in vitro studies have indicated that the CAGCTG sites function as high affinity binding sites for Daughterless + Achaete and Daughterless + Scute heterodimers (Singson et al., 1994). We tested the involvement of the *achaete–scute* in *nerfin-1* NB expression by examining *nerfin-1* expression in an *ac-sc* null background. An altered, but not complete absence, of expression, was observed indicating that either ac and/or sc TFs are direct activators of the early *nerfin-1* NB enhancer (Fig. 6). As for enhancer fragment #11, lacking bHLH sites, it contains short conserved sequence elements (Brody et al., 2007) that are found in other PNS/disc enhancers but are absent from a collection of mesodermal enhancers. Specifically, GTGGAAA, AAGGACAA and CAATGAT are conserved sequences in fragment #12, which contains the PNS enhancer: these are also present in the *scratch* PNS enhancer. The conserved elements AAAAGGG and TCGAGC are also present as conserved sequences in the *deadpan* PNS enhancer. These elements may play specific roles in PNS/disc enhancers that allow them to be activated in neuronal cells independently of proneural gene action. Further studies are needed to identify the factors binding these elements and their specific functions.

2. Experimental procedures

2.1. Comparative genomics

The multi-genome comparative analysis was carried out using the *EvoPrinterHD* program (http://evoprinter.ninds.nih.gov/). Change in reference species input DNA was performed using the appropriate link on the *EvoPrinterHD* alignment scorecard page.

2.2. Enhancer-reporter constructs

PCR primers used to amplify genomic regions for enhancer-reporter transgene analysis are described in Table 1 of the Supplemental information section. PCR was carried out using standard methods. All fragments were taken from the *nerfin-1* rescue fragment described

previously (Kuzin et al., 2005). Fragments were gel purified and cloned into the pCRII-TOPO vector. Fragments were sequenced to verify the fidelity of the PCR and recloned into pRed H-Stinger (Barolo et al., 2004). Details of our procedure are available upon request.

2.3. Drosophila P-element transformants and stocks

Germ-line transformants were generated using standard techniques based on the methodology described in Rubin and Spradling (1982). Constructs were injected into *Df(1)w67c,y*, y w, or *w118* strains using *delta 2–3* helper DNA (Rubin and Spradling, 1982). Standard procedures were used in the handling of *Drosophila* stocks (Ashburner, 1989). The *ac-sc* double mutant line (Df(1) sc10-1) was obtained from the Bloomington, Indiana *Drosophila* stock center.

2.4. Immunohistochemistry of embryos and larval tissues

Embryo fixation and whole-mount immunostaining of 8 and 16 h embryo collections were carried out according to the procedures described in Patel (1994). For staining of promoter constructs, rabbit anti-RFP obtained from Chemicon was used at a 1:1000 dilution. Vectastain ABC second antibody avidin/biotin HRP visualization reagents were used according to the manufacturer's protocol (Vector Labs). We also detected reporter expression using *in situ* hybridization to detect DsRed mRNA expression. The *in situ* ribo-probe corresponded to the full length DsRed ORF. Detailed protocols for the immunostains and *in situ* hybridization are available upon request. Embryos and dissected fillets were viewed in 70:30 ratio of glycerol to phosphate-buffered saline (PBS) and photographed using a Nikon Optiphot microscope equipped with DIC/Nomarski optics. Embryo developmental staging was determined by morphological criteria (Campos-Ortega and Hartenstein 1985). Staining of imaginal discs and larval brain was carried out as described by Mozer and Benzer (1994).

3. Conclusions

The principle findings of this study are twofold; (1) phylogenetic footprinting is an efficacious tool for identifying *cis*-regulatory DNA and (2) the lack of conservation within sequences flanking closely associated enhancers reveals their functional autonomy. By using the aligning regions of different species, *EvoPrinter* facilitates examination of the evolutionary cohesiveness of enhancers and thus reveals the lack of constrained structure within their flanking sequences. With this approach we have identified multiple enhancers that regulate the dynamic expression of *nerfin-1* during nervous system development, including enhancers that activate expression in NBs, GMC and neurons of the CNS, in nascent neurons of the PNS, and in neurons of the eye disc and optic lobe precursors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.gep.2008.10.005.

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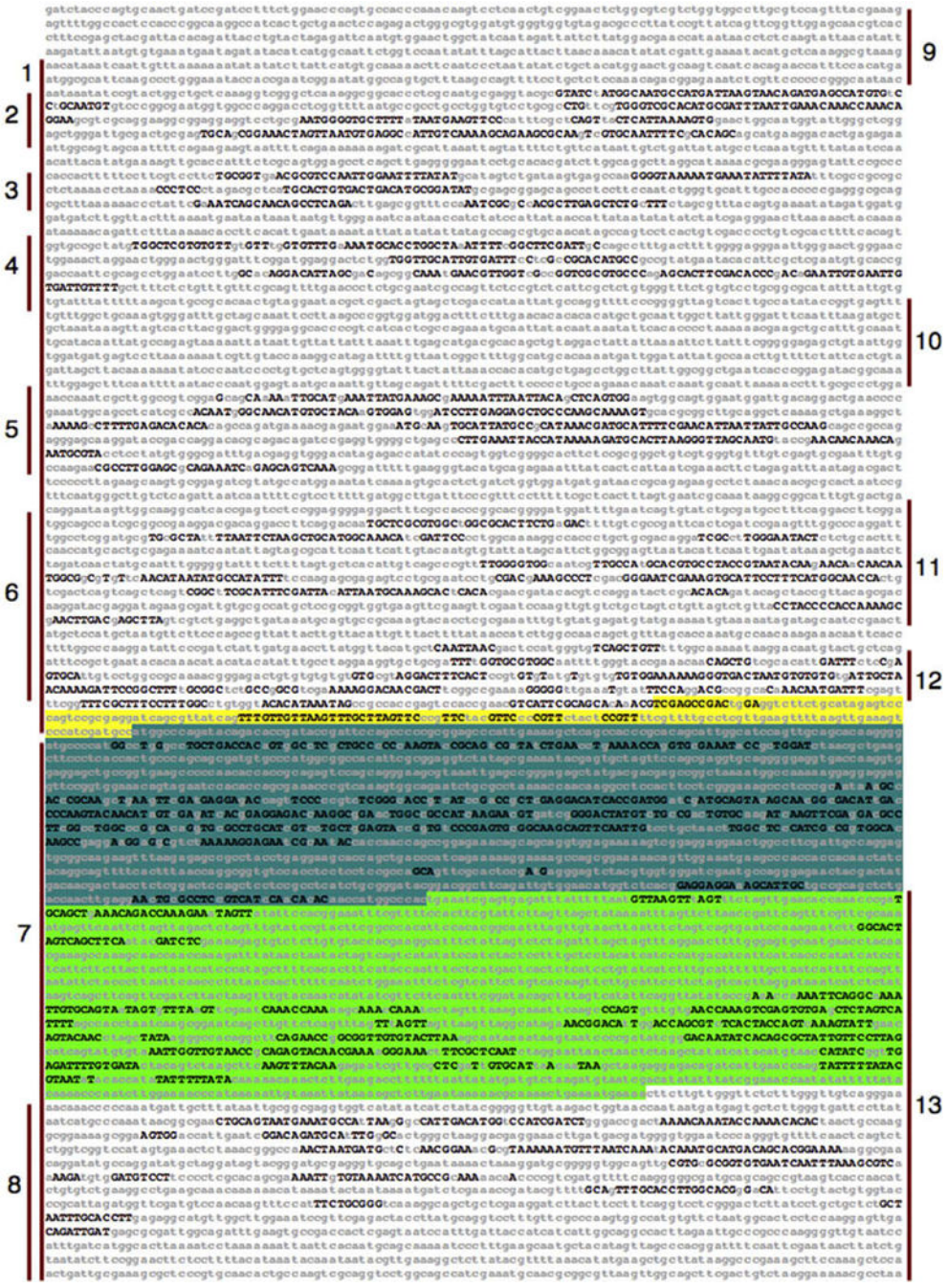

Fig. 1.

EvoPrinter analysis of the *nerfin-1* locus identifies conserved DNA clusters. Comparative genomics analysis of the *D. melanogaster nerfin-1* rescue fragment reveals DNA sequence clusters that are conserved in other drosophilids. Capital letters represent bases in the *D. melanogaster* reference sequence that are conserved in all species included in the analysis plus those that are present in all but one of the *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. virilis*, *D. mojavensis*, *D. willistoni* and *D. grimshawi* orthologous DNAs. Due to sequencing gaps in their aligning regions, *D. persimilis* and *D.*

simulans were excluded from the analysis. The colored highlighted regions indicate the *nerfin-1* transcriptional unit (5′ UTR, yellow; ORF, blue; 3′ UTR, green). The numbered bars in the margins span those DNA sequences that were tested for *cis*-regulatory function (summarized in Table 1).

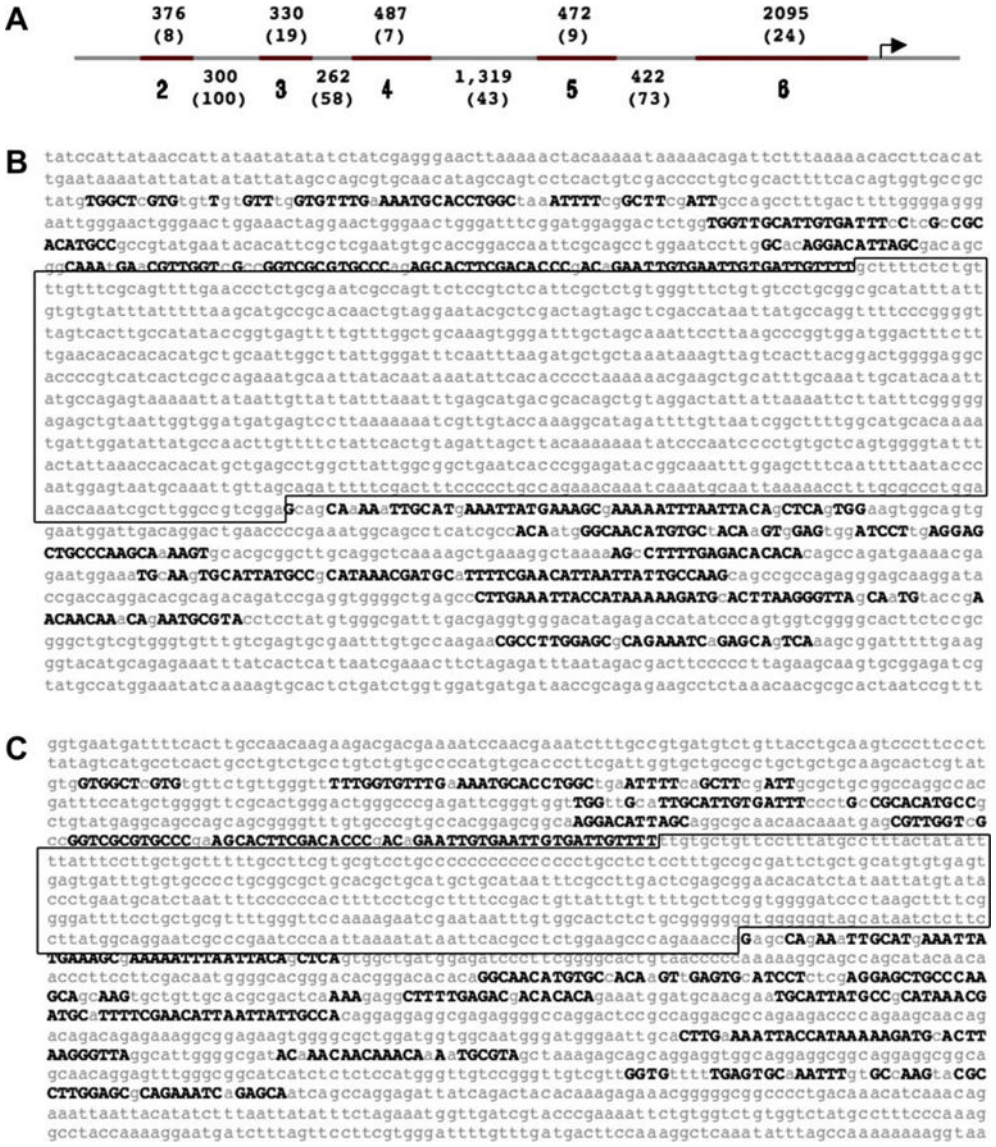

Fig. 2.

Sequence length variability between CSB clusters delineates different modular enhancers. (A) Linear cartoon of the *nerfin-1* upstream genomic DNA. Conserved sequence cluster regions identified in Fig. 1 are indicated by red-colored bars (numbered 2–6). The mean length and coefficient of variation (parentheses) are shown for both the conserved and non-conserved regions in *D. melanogaster D. pseudoobscura*, *D. willistoni*, *D. virilis*, *D. mojavensis* and *D. grimshawi*. (B and C) *EvoPrinter* analysis of the nerfin-1 upstream region that spans clusters 4 and 5 including the inter-cluster region 10 using *D. melanogaster* (B) and *D. mojavensis* (C) as reference species. Boxed sequences highlight inter-cluster regions of variable sequence length between *D. melanogaster* and *D. mojavensis*. (B) Capital letters represent bases in the *D. melanogaster* reference sequence that are conserved in the *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. willistoni*, *D. mojavensis*, *D. grimshawi and D. virilis* orthologous DNAs. (C) Capital letters represent bases in the *D. mojavensis* reference sequence that are conserved in the *D. virilis, D. grimshawi*, *D.*

willistoni, *D. simulans*, *D. melanogaster*, *D. erecta*, *D. ananassae*, *D. yakuba and D. sechellia* orthologous DNAs.

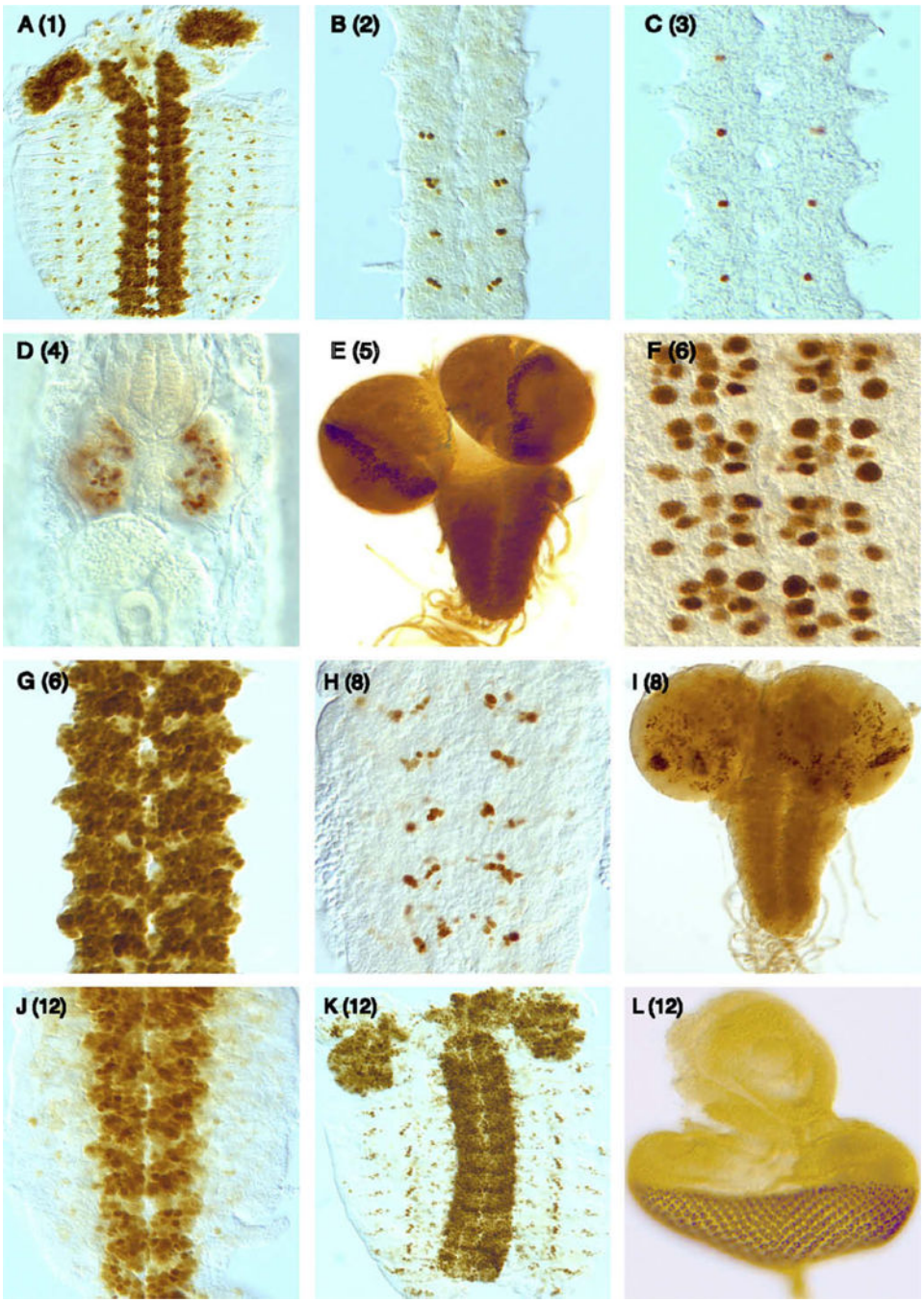

Fig. 3.

Multiple enhancers control different aspects of *nerfin-1* neural expression. Enhancer-reporter transgene analyses of different regions that span the *nerfin-1* genomic rescue fragment reveal that only those DNA fragments that contain conserved sequence clusters posses *cis*-regulatory function (summarized in Table 1). Dissected fillets of whole-mount embryos and larva tissues immunostained with anti-RFP are shown (anterior up). (A) Fragment #1, which contains all of the 5′ flanking rescue DNA, drives pan-neural reporter expression in both the CNS and PNS. Shown is a stage 13 fillet embryo. (B) Fragment #2 activates expression

during stage 14 in a subset of abdominal segment ventral cord neurons but not in thoracic segments. (C) Dissected ventral cord from a stage 13 embryo reveals that fragment #3 contains *cis*-regulatory elements that drive expression in another subset of neurons that reside in both the thoracic and abdominal segments. (D) Fragment #4 activates reporter expression in a subset of cephalic lobe neurons during stage 15 (shown is a dorsal view of a whole-mount embryo). (E) Fragment #5 does not have embryonic *cis*-regulatory activity. However, it activates reporter expression in a subset of cephalic and ventral cord neurons in the third-instar CNS. (F and G) Fragment #6 directs expression in early delaminating stage 9 CNS neuroblasts (F), in GMCs and in most nascent CNS neurons (G), shown in a dissected stage 13 ventral cord. (H and I) The 3′ flanking genomic fragment #8 activates expression in a subset of stage 11 NBs and their GMCs (H), and in a subset of neurons within the third-instar larva brain (I). J–L) Fragment #12 activates reporter expression in ventral cord GMCs and neurons (J; stage 12), and expression is detected in both CNS and PNS neurons (K; stage 14). Fragment #12 also activates expression in third-instar eye disc photoreceptor neurons (L).

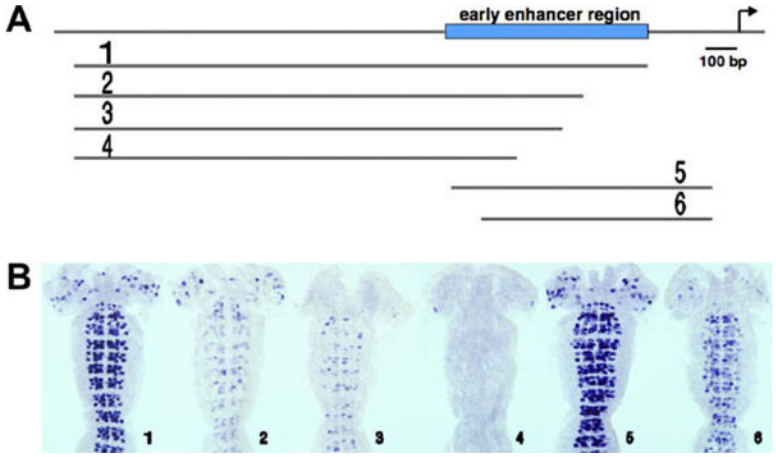

Fig. 4.

Localization of the *nerfin-1* early neuroblast enhancer. (A) Truncation analysis of 1.8 kb fragment #6, which activates reporter expression in the early delaminating S1 and S2 NBs, GMCs and CNS neurons, revealed that the NB enhancer corresponded to a 567 bp region located 249 bp upstream of the *nerfin-1* transcription start site. The truncated fragments used to map the NB enhancer are aligned with a linear cartoon of the 5′ flanking region. The blue-colored box indicates the location of the NB enhancer (arrow indicates the predicted transcription start site). (B) Enhancer-reporter transgene expression analysis (DsRed mRNA *in situ* hybridization) of the different sub-regions. Dissect fillets of whole-mount stage 9 embryos are shown (anterior is up). Numbers correspond to the sub-regions shown in panel A. Note that only sub-regions 1 and 5 activated reporter expression in a manner similar to *nerfin-1* wild-type NB expression.

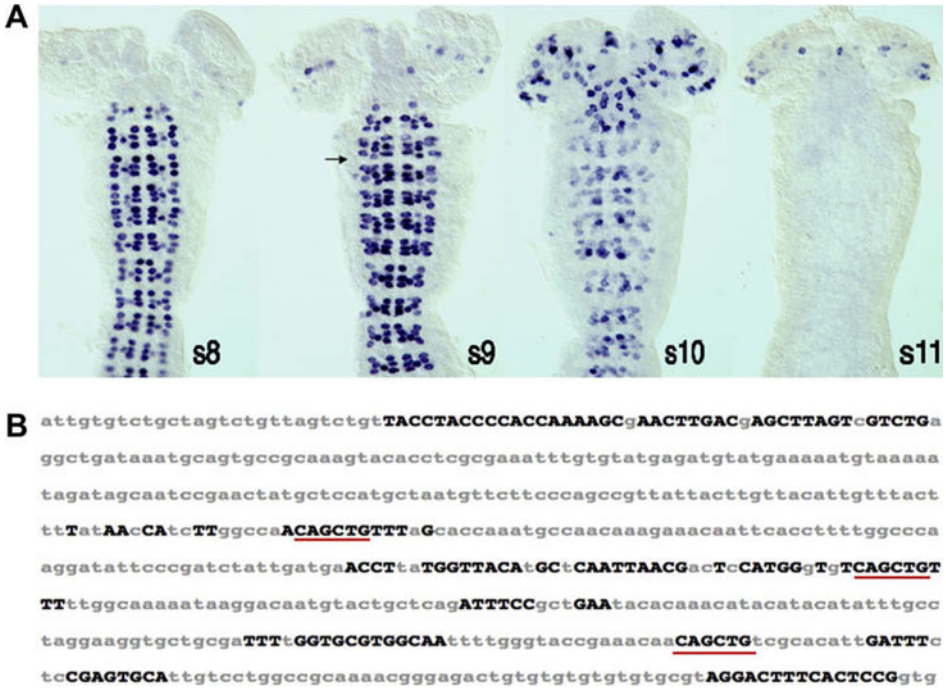

Fig. 5.

Dynamic expression and sequence conservation within the *nerfin-1* early neuroblast enhancer. (A) Expression dynamics of *nerfin-1* neuroblast enhancer revealed by DsRed mRNA *in situ* hybridization. Shown are filleted embryos, stages 8 through 11 (s8–s11; anterior up). Arrow indicates the lack of expression in row one NBs. (B) An *EvoPrint* of the *D. melanogaster* NB enhancer sequence reveals that it contains multiple conserved sequence blocks. Potential bHLH TF binding sites are underlined. Uppercase capital letters in the *D. melanogaster* reference sequence represents conserved bases in all or all but one of the following species; *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura* and *D. virilis*.

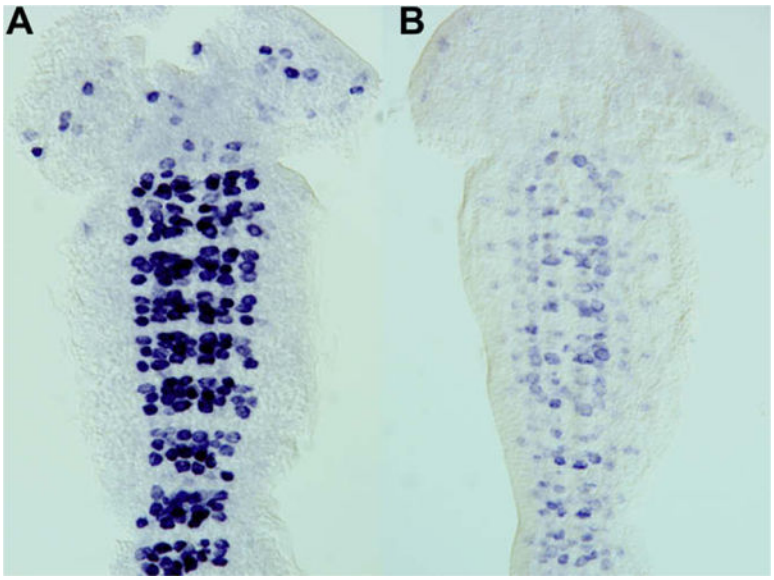

Fig. 6.

Achaete–Scute transcription factors are required for full *nerfin-1* neuroblast enhancer activity. *nerfin-1* NB expression in late stage 9 wild-type (A) and *achaete–scute* deficient (B) embryos. Embryos were subject to whole-mount *in situ* hybridizations to reveal endogenous *nerfin-1* mRNA expression. Shown are flattened filleted embryos (anterior up). Note the significantly lower level of endogenous *nerfin-1* expression in the *ac-sc* double mutant embryo.

Table 1

Expression summary of *nerfin-1 cis*-regulatory-reporter transgenes.

Construct	Transformant lines[a]	Embryo	Larva[b]	Fig.
#1	4	NPCs and neurons in CNS and PNS	NPCs, neurons in the CNS, PNS and eye disc	3A
#2	4	Subset of CNS Neurons	No expression	3B
#3	4	Subset of CNS Neurons	No expression	3C
#4	3	Subset of CNS neurons	No expression	3D
#5	3	No expression	Subset of CNS neurons	3E
#6	3	CNS NBs, GMCs and neurons	CNS neurons	3F and 3G
#7	3	Same as #8	Same as #8	Not shown
#8	3	Subset of CNS NBs	CNS neurons optic lobes	3H and I
#9	2	No expression	No expression	Not shown
#10	2	No expression	No expression	Not shown
#11	5	No expression	Cephalic lobe neurons	Not shown
#12	5	GMCs and neurons in CNS and PNS	NPCs, neurons in the CNS, PNS and eye disc	3J, K and L
#13	3	Same as #8	Same as #8	Not shown

[a] Number of independent transformant lines tested.

[b] Excluding vector-dependent salivary gland expression (see Qianqian and Halfon, 2007).