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Identification of *hunchback* cis-regulatory DNA conferring temporal expression in neuroblasts and neurons

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ABSTRACT

The specification of temporal identity within single progenitor lineages is essential to generate functional neuronal diversity in Drosophila and mammals. In Drosophila, four transcription factors are sequentially expressed in neural progenitors (neuroblasts) and each regulates the temporal identity of the progeny produced during its expression window. The first temporal identity is established by the Ikaros-family zinc finger transcription factor Hunchback (Hb). Hb is detected in young (newly-formed) neuroblasts for about an hour and is maintained in the early-born neurons produced during this interval. Hb is necessary and sufficient to specify early-born neuronal or glial identity in multiple neuroblast lineages. The timing of *hb* expression in neuroblasts is regulated at the transcriptional level. Here we identify cis-regulatory elements that confer proper *hb* expression in "young" neuroblasts and early-born neurons. We show that the neuroblast element contains clusters of predicted binding sites for the Seven-up transcription factor, which is known to limit *hb* neuroblast expression. We identify highly conserved sequences in the neuronal element that are good candidates for maintaining Hb transcription in neurons. Our results provide the necessary foundation for identifying trans-acting factors that establish the Hb early temporal expression domain.

Temporal patterning is particularly important for central nervous system (CNS) development, due to the vast number of cell types that need to be produced by relatively few progenitors. In the mammalian CNS, individual neural progenitors give rise to an ordered series of cell types in the cerebral cortex, retina, and spinal cord (reviewed in Pearson and Doe, 2004). In Drosophila, embryonic neuroblasts (NBs) undergo a series of asymmetric cell divisions to generate smaller progeny called ganglion mother cells (GMCs) that typically produce a pair of neurons (Doe, 2008). We and others have identified four transcription factors – Hunchback (Hb), Krüppel (Kr), Pdm, Castor (Cas) – that are sequentially expressed in embryonic NBs and maintained in the GMC and neuronal progeny born during the expression window (Brody and Odenwald, 2000; Isshiki et al., 2001; Kambadur et al., 1998). The early factor Hb is necessary and sufficient to specify the fate of early-born neurons in multiple NB lineages (Cleary and Doe, 2006; Grosskortenhaus et al., 2005; Isshiki et al., 2001; Kanai et al., 2005; Novotny et al., 2002; Pearson and Doe, 2003; Tran and Doe, 2008).

Genetic experiments using Gal4 lines to drive *UAS-hb* expression beyond its normal window of expression in NBs leads to overproduction of early-born neuronal fates and a delay or loss of the later-born neuronal fates in all tested NB lineages (Cleary and Doe, 2006; Isshiki et al., 2001; Kanai et al., 2005; Mettler et al., 2006; Novotny et al., 2002; Pearson and Doe, 2003). Thus, it is critical to downregulate *hb* expression to generate neuronal diversity. A study of *hb* neuroblast expression patterns using (a) intron probes to detect active transcription, (b) coding sequence probes to detect cytoplasmic mature mRNA, and (c) antibody probes to detect protein revealed a strong congruence among all three probes, indicating that the timing of *hb* neuroblast expression is primarily regulated at the transcriptional levels (Grosskortenhaus et al., 2005). Furthermore, experiments using *in vitro* cultured NBs show that lineage-extrinsic cues are not required for *hb* downregulation (Grosskortenhaus et al., 2005); however, blocking cytokinesis will prevent *hb* downregulation (Grosskortenhaus et al., 2005). These cellular studies suggest that repression of *hb* requires either the segregation of regulatory factors out of the NB during asymmetric cell division, or "feedback" signaling from the newborn GMC to the NB. In addition, the orphan steroid hormone receptor Seven-up (Svp) is required for timely repression of *hb* expression (Kanai et al., 2005; Mettler et al., 2006), as is the

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Pipsqueak domain nuclear protein Distal antenna (Dan) (Kohwi et al., 2011). Recently it has been shown that the transcriptional repressor Prospero is required to keep Svp levels low (Mettler et al., 2006); Prospero is normally segregated out of the NB into the GMC (Spana and Doe, 1995), which may explain why NBs that do not undergo cytokinesis maintain *hb* expression: they have nuclear Prospero which prevents accumulation of Svp and thus allows persistent *hb* expression. This attractive model remains to be tested.

To understand the temporal regulation of *hb* expression in NBs, we have identified the cis-regulatory DNA that confers proper temporal expression of *hb* in NBs, and identified additional cis-regulatory elements that promote *hb* expression in early-born neurons and repress *hb* expression in late-born neurons. In addition, we have found an unexpected cis-regulatory element that drives expression only in late-born neurons, where *hb* is normally never detected.

1. Results and discussion

1.1. Generation of hb enhancer-reporter constructs

During previous studies of the *hb* cis-regulatory region controlling early blastoderm expression (Margolis, 1992; Margolis et al., 1994; Margolis et al., 1995), we discovered an ~4 kb element that conferred expression in neuroblasts and GMCs but not neurons (HZ4 element; Fig. 1A). Here, we extend our analysis to subdivide this 4 kb element using a series of *hb-green fluorescent protein* (*hb-GFP*) constructs containing (from 5′ to 3′) *hb* cis-regulatory DNA, the *hb* P1 5′ UTR including the first intron and the P2 promoter, a basal *hsp70* promoter, *GFP* coding sequence, the *hb* 3′ UTR, the SV40 transcription termination sequence, and an attB sequence (Fig. 1B). We then used the PhiC recombinase system to insert all transgenes into the same genomic site on chromosome 2. This eliminates variability in transgene expression due to chromosomal position effects, which can be a problem when inserting transgenes randomly in the genome. We used EvoPrinterHD software (Odenwald et al., 2005; Yavatkar et al., 2008) to identify blocks of evolutionarily-conserved sequence within the largest construct (HG4-1; Fig. 2), and then designed smaller constructs centered around these blocks of conservation (HG4-2 through HG4-7) as shown in Figs. 1 and 2. We originally designed the constructs using a more restrictive EvoPrint showing sequences conserved among all twelve Drosophila species, but present the EvoPrint of sequences conserved among 11/12 species. This resulted in a small block of conservation between HG4-6 and HG4-7 not being included in these smaller constructs. This region of DNA could contain regulatory information that might help sum HG4-6 and HG4-7 to match the larger HG4-3 construct.

1.2. Identification of hb cis-regulatory DNA conferring proper temporal expression in NBs

We performed RNA in situ hybridization with an antisense GFP probe to whole mount embryos expressing each of the *hb-GFP* reporter transgenes (Fig. 3). The endogenous *hb* gene (detected with a *hb*-specific probe) shows maximal expression in NBs during stages 9 and 10 (Fig. 3, NB layer marked with black arrows). Importantly, expression in the neuroblast layer is lost by stage 11 (Fig. 3, black arrows), although there is clear expression in early-born GMCs at this stage (Fig. 3, white arrowheads). Using a GFP probe, we found that the HG4-1, HG4-3, and HG4-7 transgenes are all expressed in a pattern similar to endogenous *hb*: expression in NBs at stage 9 and stage 10, followed by early-born GMC expression at stage 11. Although these transgenes may have been expressed at lower levels than endogenous *hb* (or the probes were less effective), it is important to note that all three transgenes showed the proper temporal pattern. The remaining HG transgenes (HG4-2, HG4-4, HG4-5, HG4-6) did not show NB expression (Fig. 3).

We do not show ventral views of the transgene RNA patterns in Fig. 3 because the expression of endogenous *hb*, and the transgenes, is transient in neuroblasts (at the time they form), and neuroblasts form at different times from the earliest at stage early 9 (e.g. 7–1) to the latest at late stage 11 (e.g. 7–3). Thus, the RNA in situ's will never show the full complement of neuroblasts at a single time point. Instead, to determine if the three HG transgenes with proper temporal expression also had proper spatial expression within the NB population, we stained for GFP and the neuroblast marker Deadpan (Dpn) and imaged the field of NBs; we used the positional marker Engrailed to determine segment boundaries (Fig. 4). Because GFP has a relatively long half-life, this experiment allowed us to "sum up" the expression of the HG transgenes during stages 9 and 10 to determine if they were properly expressed in the majority of NBs in each segment. We found that the HG4-1, HG4-3, and HG4-7 transgenes each expressed in >90% of NBs per segment (Fig. 4), similar to endogenous Hb protein (Isshiki et al., 2001). We conclude that the nested DNA elements contained in HG4-1, HG4-3, and HG4-7 all possess the cis-regulatory DNA necessary for proper temporal expression in newly-formed NBs; this maps the element to the smallest transgene DNA, HG4-7, which contains 961 base pairs. We conclude that a 961 nucleotide cis-regulatory DNA element is sufficient to generate the *hb* temporal expression pattern within embryonic NBs.

Interestingly, this region of DNA contains seven predicted Svp binding sites (AGGTCA or its complement, with one mismatch allowed; Fig. 2, blue highlighting), including one pair spaced 10 nucleotides apart that is predicted to allow Svp homo- or heterodimer binding (Zelhof et al., 1995); this site is not detected in any other conserved sequence blocks within the 4 kb HG4-1 region. In addition to the predicted Svp binding sites, which may

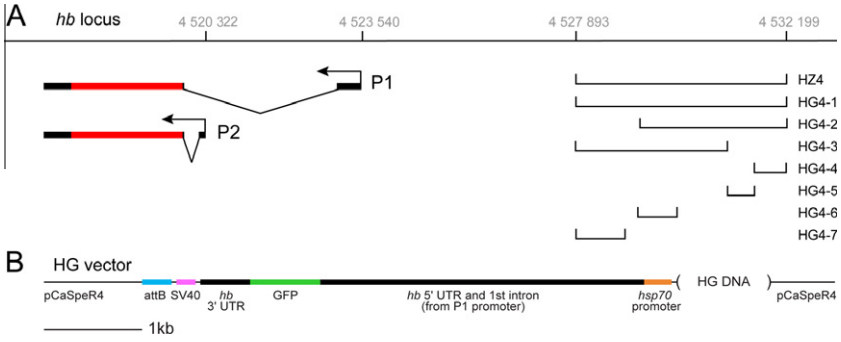

Fig. 1. Location of enhancer-reporter constructs at the *hunchback* (*hb*) locus. The two *hb* transcripts produced from the P1 and P2 promoters are shown (black, untranslated regions; red, coding region). Gray numbers at top are the Flybase coordinates for the region. Lines shown at right indicate the DNA elements used in the reporter constructs (see methods for construct details). Each of these DNA elements lie within the first intron of CG8112, whose promoter start site is 4526704 on the strand opposite that of *hb*.

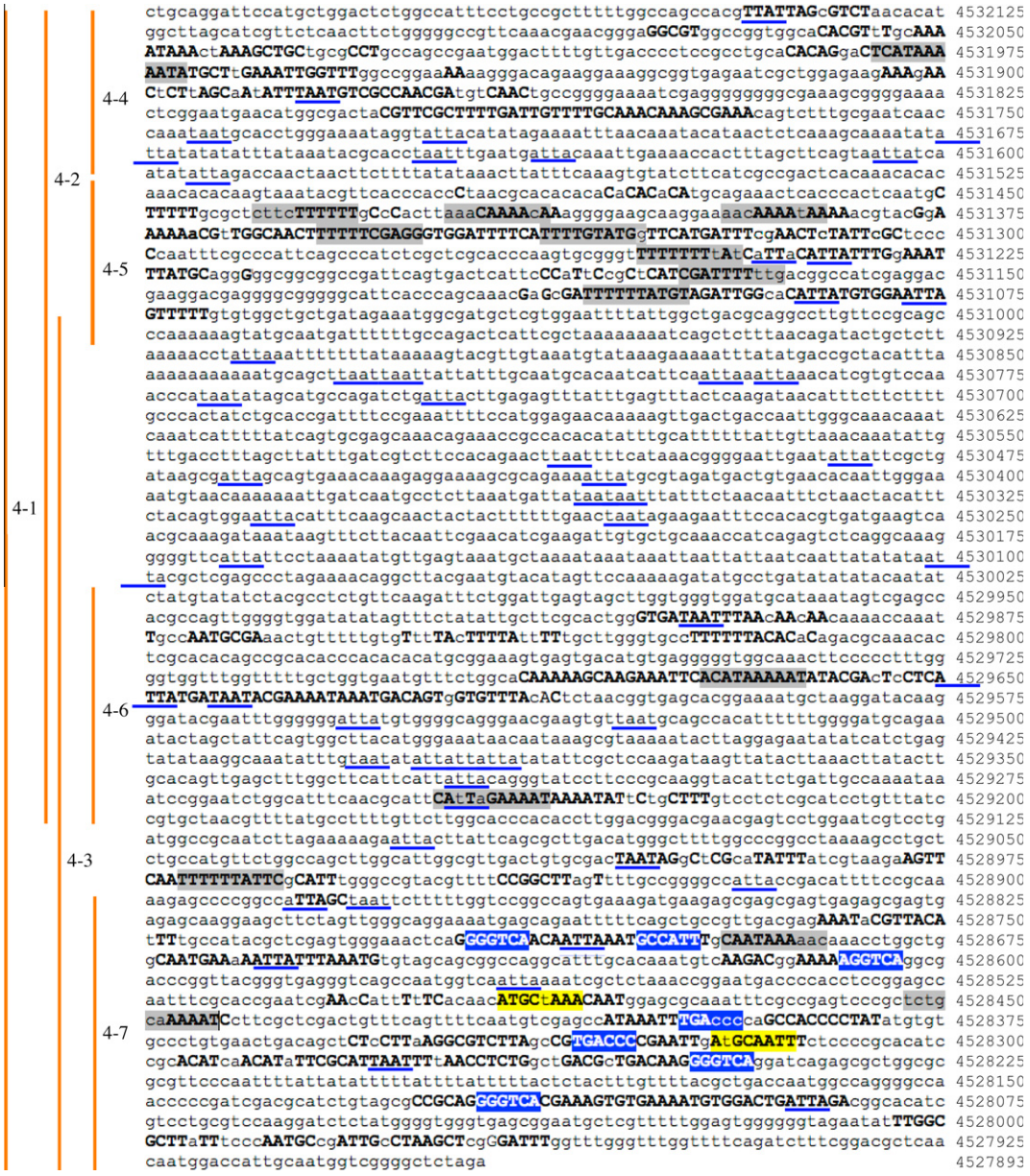

Fig. 2. Evoprint of the cis-regulatory DNA used in this study. Bold capital letters represent conservation in either all 12 or 11/12 Drosophila species for which genomic data exists. The Flybase coordinates for the last nucleotide of each row is shown to the right, and the approximate positions of the constructs used is shown to the left (see Supplemental data Table 1 for primers used in these constructs). Transcription factor predicted binding sites within the conserved blocks of DNA were determined using the TESS website (http://www.cbil.upenn.edu/cgi-bin/tess). Blue, Seven-up (Svp) binding sites; gray, Hb binding sites; yellow, Pou domain binding sites, including Nubbin/Pdm2; blue underlines, homeodomain core motifs (ATTA/TAAT) sites.

be important for limiting Hb expression, there are other blocks of conserved sequence within the HG4-7 DNA that may represent binding sites for direct or indirect Dan-mediated repression of *hb* transcription (Kohwi et al., 2011), or binding sites for currently unknown transcriptional activators of *hb* expression in NBs. Mutation of individual small blocks of conserved sequence would be necessary to test these hypotheses; sites found to be functionally important would be excellent candidates for biochemical purification and identification of trans-acting factors that regulate the timing of *hb* expression in neuroblasts.

1.3. Identification of hb cis-regulatory DNA conferring proper expression in early-born neurons

We next examined embryos at stage 16 to determine the expression pattern of each transgene in post-mitotic neurons. At this stage, early-born neurons are located in the deepest layer of the CNS, whereas later-born neurons occupy a more superficial position (Isshiki et al., 2001). The endogenous *hb* gene is weakly detected in deep layer neurons in lateral views (Fig. 5, top row). Using an antisense GFP probe, we found that the HG4-1, HG4-2, HG4-3, and HG4-6 transgenes are all expressed in deep layer neurons (Fig. 5); in addition, HG4-5 appears to be expressed in deep layer neurons and strongly expressed in superficial (late-born) neurons (Fig. 5). The transgenes HG4-4 and HG4-7 show little or no RNA expression in the stage 16 CNS (Fig. 5). Lower magnification ventral views of stage 16 embryos show the relative level of RNA in the CNS generated by each transgene (Fig. 5, right column). The observation of neuronal expression for the HG4-1 construct was somewhat surprising, as the HZ4 transgene did not show neuronal expression (data not shown). This could be due to position effects acting on the HZ4 transgene (which was randomly

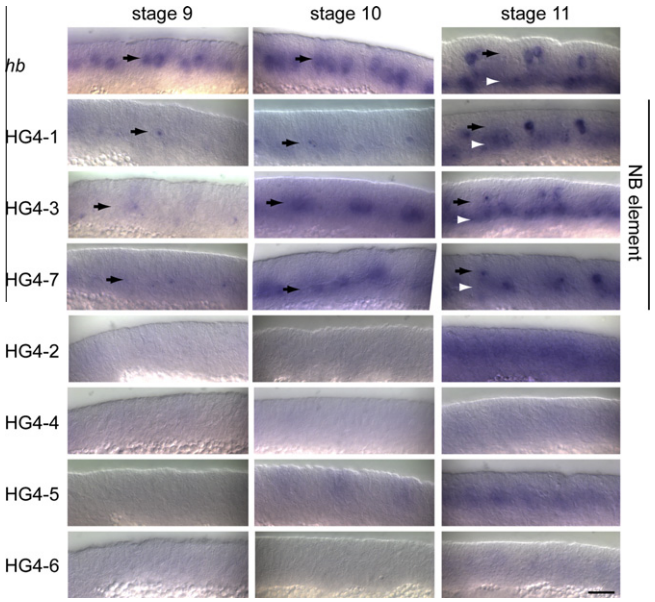

Fig. 3. Expression of the endogenous *hunchback* gene (*hb*) and enhancer-reporter transgenes (HG series) in neuroblasts and GMCs. Drosophila embryonic NBs first delaminate at early stage 9 and express *hb* at the time of their delamination; they maintain *hb* for about an hour until stage 10. In addition, some NBs first delaminate at stage 10 or stage 11, and they also typically express *hb* for about an hour after delaminating, so that there are scattered Hb+ NBs persisting into stage 11. Nevertheless, the bulk of *hb* expression occurs at stage 9 and 10. RNA in situ hybridization shown for the indicated genotypes. Black arrows indicate the neuroblast layer in stage 9–11 embryos; white arrowheads indicate the GMC layer in the stage 11 embryos. Only lines HG4-1, HG4-3, and HG4-7 show neuroblast expression ("neuroblast element" label, to right). Scale bar, 20 μm.

inserted into the genome, as opposed to placed at same attP genomic site like all the HG transgenes), or due to the lack of regulatory sequences mapping to the *hb* 5′ or 3′ UTR (which were not present in the HZ4 construct).

To determine the expression pattern of each transgene within early-born and late-born neurons of the CNS at higher resolution, we double stained each of the transgenic lines for GFP and Hb protein (a marker for deep-layer, early-born neurons). We found that HG4-1, HG4-2, HG4-3, and HG4-6 transgenes all showed GFP staining in deep layer neurons (Fig. 6A–G), although it was not a perfect 1-to-1 register with the Hb + deep layer neurons. Some GFP + neurons were in Hb-negative neurons, whereas some Hb + neurons lacked GFP. For example, line HG4-6 had Hb + neurons that were 19% strong GFP, 37% weak GFP, 44% no GFP ($n = 328$ neurons from two embryos); the lack of GFP in some Hb + neurons is most likely due to lack of full cis-regulatory information in the transgene, but we can't rule out low levels of GFP being present. In addition, HG4-5 showed strong GFP staining in Hb-negative superficial neurons (Fig. 6K), whereas the other transgenes were weakly or not detected in superficial neurons (Fig. 6H–J, L–N); see next section. Unexpectedly, we noticed HG4-7 exhibited a clear GFP protein signal within the CNS (Fig. 6G and N) despite lack of detectable GFP RNA at this stage (Fig. 5). The GFP protein may be due to persistence of GFP from neuroblasts and GMCs into the neurons. We conclude that the smallest DNA element that drives early-born neuron expression is the 866 base pair HG4-6 element, and that all larger elements containing this fragment also show early-born neuron expression.

It is currently unknown how *hb* expression is maintained in the GMCs and neurons born from Hb+ young neuroblasts. We can rule out simple positive autoregulation because misexpression of Hb in neurons does not activate *hb* transcription (Grosskortenhaus et al., 2005). The presence of several blocks of highly conserved sequence within the 866 base pair HG4-6 element would be excellent starting points for identifying the transcriptional activator(s) required to maintain Hb expression in early-born neurons.

1.4. Identification of a cis-regulatory DNA element conferring expression in late-born neurons

The HG4-5 showed strong reporter expression in Hb-negative superficial neurons (Fig. 6K), which was surprising because Hb is not detected in these neurons at any embryonic stage (Isshiki et al., 2001; Kambadur et al., 1998). None of the other transgenes were strongly expressed in these neurons (Fig. 6H–J, L–N), although we detected weak "stripy" GFP reporter expression in the superficial layer neurons in line HG4-7 (Fig. 6N). To test whether HG4-5 was preferentially expressed in late-born neurons, we assayed lateral confocal sections through the stage 16 CNS double stained for GFP and Hb protein. We observed a virtually complementary pattern of GFP and Hb expression (Fig. 6O–Q). This shows that the DNA contained in the HG4-5 transgene contains an enhancer that drives expression in late-born neurons. Interestingly, the HG4-2 construct encompasses the HG4-5 fragment but does not have similar strong expression in late-born neurons (compare Fig. 6I with 6 K); this shows that HG4-2 must contain a late-born neuron silencer element. We do not know whether the late-born neuron enhancer found in HG4-5 is normally silenced by adjacent regulatory elements during wild type development, or if it drives expression of a flanking gene such as *CG8112*, whose promoter is closer to the HG4-5 element than the *hb* promoter (*CG8112* is transcribed in the opposite direction as *hb*, and its predicted promoter lies between the HG4-5 element and the *hb* promoters; i.e. the HG4-5 element is in the first intron of *CG8112*). Nevertheless, the serendipitous identification of a late-neuron enhancer will be useful as a tool (e.g. making a Gal4 line with late-born neuron expression);

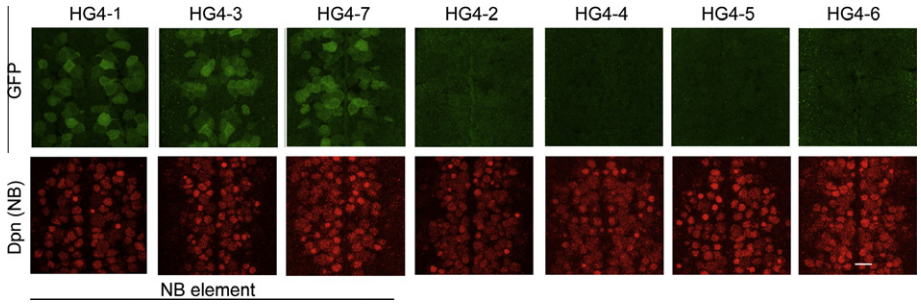

Fig. 4. Expression of *hb* enhancer-reporter transgenes (HG series) in stage 11 neuroblasts. Confocal imaging of embryos stained for GFP (top row) and the neuroblast marker Deadpan (Dpn, bottom row). Lines HG4-1, HG4-3, and HG4-7 show expression in most or all neuroblasts ("neuroblast element" label, at bottom). Two segments are shown, determined by Engrailed staining (not shown). Scale bar, 10 μm.

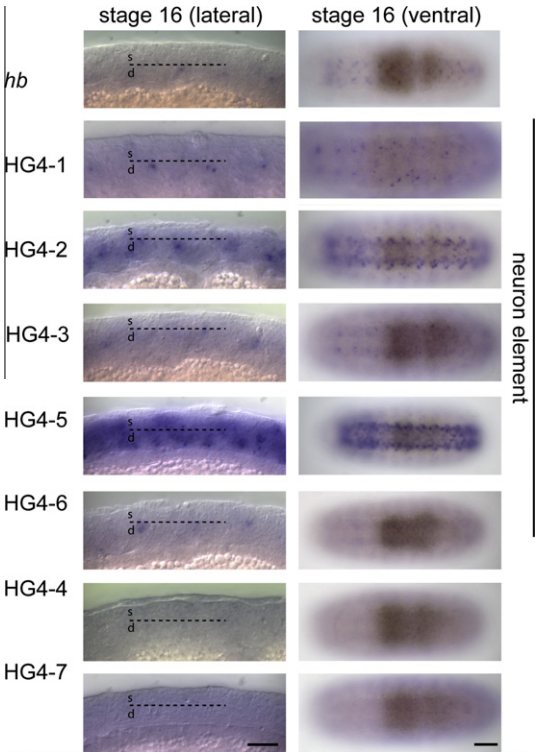

Fig. 5. Expression of the endogenous *hunchback* gene (*hb*) and enhancer-reporter transgenes (HG series) in neurons of stage 16 embryos, shown in lateral (left) or ventral (right) views. RNA in situ hybridization shown for the indicated genotypes. In the left column, deep neurons (d) are located below the dashed line and superficial neurons (s) are located above the dashed line. Lines HG4-1, HG4-2, HG4-3, HG4-5 and HG4-6 show neuronal expression ("neuron element" label, to right). Scale bar, 20 μm (left column) and 50 μm (right column).

future experiments will be necessary to determine its relevance to *hb* or *CG8112* transcription.

HG4-5 is only 571 base pairs long, and contains just two blocks of conserved sequence. Interestingly, there are eight predicted Hb binding sites within these small blocks of conserved sequence, raising the possibility that Hb acts as a transcriptional auto-repressor to prevent the function of this enhancer fragment within early-born neurons (e.g. to keep the adjacent *CG8112* gene off in early-born neurons). In this case, we might expect the remaining small region of conserved sequence to contain binding site(s) for a pan-neuronal transcriptional activator.

2. Experimental procedures

2.1. Enhancer-reporter transgenic lines and other stocks

The HZ4 construct was made as described previously (Margolis, 1992; Margolis et al., 1994; Margolis et al., 1995); briefly, it contains the DNA from Flybase position 4527893 (*Xba*I site) to 4532199 (*Pst*I site) – coordinates shown in Fig. 1A – attached to a basal *hsp70* promoter directly upstream of the *lacZ* coding sequences. Unlike the HG constructs described below, there is no *hb* 5′ or 3′ UTR sequences in the HZ4 construct; furthermore HZ4 was randomly inserted into the genome. The HG4-1 construct contains precisely the same upstream 4 kb of DNA as the HZ4 construct, but driving a different reporter (GFP instead of lacZ). All HG constructs are summarized in Fig. 1A (different cis-regulatory fragments) and Fig. 1B (core construct that is common to all HG transgenes). To make the core HG construct, *hb* 5′UTR was amplified from a BAC clone, BACR01F13 using forward primer 5′CAGTTGTGCTCCGAGTCC and reverse primer 5′CTCGCCCTTGCTC ACCATCTTGGCGGCTCTAGACGG, which contained *GFP* 5′ coding sequence. The *hb* 3′UTR was amplified from BACR01F13 using forward primer 5′GCATGGACGAGCTGTACAAGTAAGTTCCCCATCACC ATCACCTTG, which contained *GFP* 3′ coding sequence and reverse primer 5′TATATTGAATAATTGGATTTATTTGATTTGATTTCGTTC. We inserted the GFP coding sequence between the *hb* 5′ UTR and 3′ UTR by assembly PCR utilizing forward primer 5′CCGTCTAGAG CCGCCAAGATGGTGAGCAAGGGCGAG, which contained *hb* 3′ 5′UTR sequence and reverse primer 5′CAAGGTGATGG TGATGGGGAACT-TACTTGTACAGCTCGTCCATGC, which contained *hb* 5′ 3′UTR sequence. This core *hb-gfp* gene was cloned into *Spe*I-*BamH*I site of a modified pCaSpeR4 vector by inserting a basal *hsp70* promoter (hsp70 TATA box) at *Not*I-*Spe*I site, SV40 termination signal at *Xba*I-*Hpa*I site, and attB site at *Xho*I site to make the core HG vector shown in Fig. 1B.

Using EvoPrinterHD software (Odenwald et al., 2005; Yavatkar et al., 2008), we found 4 regions evolutionally conserved in Drosophila melanogaster plus all 11 other Drosophila species, which was used to design fragments; a slightly more relaxed EvoPrint of D. melanogaster and 10/11 other species is shown (Fig. 2). Enhancer fragments were amplified from BACR01F13 using primers described in Supplemental data Table 1 and cloned into *Kpn*I-*Not*I site of HG vector. All transgenes were inserted into the same attP1 docking site on chromosome 2 by GenetiVision (Houston, TX). *yellow white* was used as wild type.

2.2. RNA in situ hybridization to embryos

Embryo whole-mount in situ RNA hybridization was carried out as previously published (Tautz and Pfeifle, 1989). The *hb* RNA probe was generated from BACR01F13 using forward primer 5′GTTCCCCATCACCATCACCTTG and reverse primer 5′CGTTCGATT CGAATTCGCTTTC with a T7 promoter sequence. For the *GFP* RNA probe, we used forward primer 5′CTTCTTCAAGTCCGCCATGC and reverse primer 5′AACTCCAGCAGGACCATGTG with a T7 promoter sequence. DIG Labeling Kit (Roche Diagnostics, Indianapolis, IN) was used for transcription and digoxygenin-labeling. Images were photographed by Zeiss AxioCam HRc camera on an Axioplan microscope DIC/Nomarski optics.

2.3. Immunofluorescence and DIC

Antibody staining was performed according to standard methods (Kohwi et al., 2011). Primary antibodies, dilutions and sources were: chicken anti-GFP 1:500 (Aves Labs, Inc., Tigard, OR, USA); rabbit anti-Hb 1:200 (Tran and Doe, 2008); mouse anti-Even-skipped (3C10-c) and mouse anti-Engrailed (4D9) (Developmental Studies Hybridoma Bank, University of Iowa, IA, USA); rat anti-Dpn (Doe lab). Donkey anti-chicken Dylight 488- and donkey anti-mouse Dylight 649-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA USA). Goat anti-rabbit Alexa Fluor 555- and Goat anti-rat Alexa Fluor 555-conjugated secondary antibodies were from Invitrogen (Eugene, OR USA). Confocal image stacks were collected using Zeiss LSM 700 confocal microscope, processed using ImageJ (NIH), and assembled into figures using Illustrator and Photoshop (Adobe Systems Inc., Mountain View, CA, USA).

2.4. Transcription factor binding site identification

Transcription Element Search System software (TESS; http://www.cbil.upenn.edu/cgi-bin/tess) was used to identify predicted transcription factor binding sites within the conserved evoprinted regions shown in Fig. 2. The Svp sites were annotated as CF1/USP sites, but these are equivalent to Svp sites (Zelhof et al., 1995).

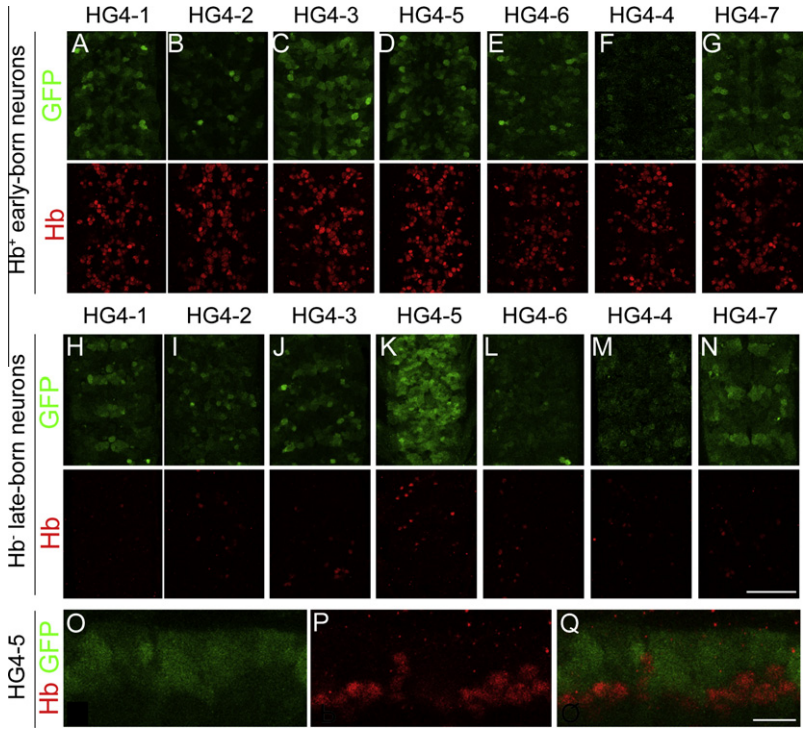

Fig. 6. Expression of the endogenous *hunchback* gene (*hb*) and enhancer-reporter transgenes (HG series) in the stage 16 CNS. Confocal images of the CNS stained for HG reporter construct patterns (GFP, green) and endogenous Hb protein (red). (A–G) Ventral view of deep layer, early-born, Hb+ neurons. Lines HG4-1, HG4-2, HG4-3, HG4-5, HG4-7, and HG4-6 show expression in these Hb+ neurons. (H–N) Ventral view of superficial layer, late-born, Hb-negative neurons. Only line HG4-5 shows strong expression in these Hb-negative neurons; other lines show scattered expression (HG4-1, HG4-2, HG4-3) or high background due to increasing the confocal gain to try and detect expression (HG4-4, HG4-6, HG4-7). For A–N, two segments are shown, determined by Even-skipped staining (not shown). (O–Q) Lateral view of a stage 16 CNS showing HG4-5 expression in superficial neurons (O, Q; green GFP staining) and almost no expression in Hb+ deep layer neurons (P, Q; red endogenous Hb staining). Scale bar is 50 μm in A–N, and 10 μm in O–Q.

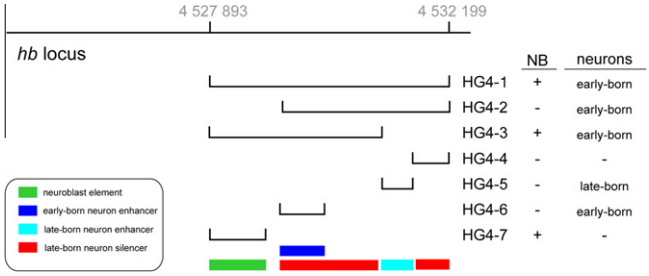

Fig. 7. Summary of the cis-regulatory elements controlling *hunchback* (*hb*) CNS expression. Gray numbers at top are the Flybase coordinates for the region. Lines shown at right indicate the DNA elements used in the reporter constructs. Color code for the four different regulatory functions discovered in this work are shown in the box, lower left.

We only indicate high confidence sites; we excluded low information content sites under 5 base pairs. Where there were overlapping sites for a single factor, the site with the highest TESS score was used.

3. Conclusions

We conclude that the pattern of *hb* expression in the CNS can be recapitulated in large part by the cis-regulatory DNA contained in the ~4 kb HG4-1 construct. Within this stretch of DNA are contained smaller elements: a 961 bp element that confers proper *hb* expression in newly-formed NBs (HG4-7), a 866 bp element that confers proper *hb* expression in early-born neurons (HG4-6), and an unexpected 571 bp element that confers expression in *hb*-negative late-born neurons (HG4-5); summarized in Fig. 7. The NB element contains predicted binding sites for a known trans-acting factor that regulates *hb* NB expression (Svp) and several other conserved regions that may represent transcriptional activator binding sites. The results of our study will provide a foundation for future functional analysis of this temporal cis-regulatory DNA, and the identification of trans-acting factors that control the precise timing of *hb* expression in the CNS.

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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.2011.10.001.

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