Specification of Motoneuron Fate in *Drosophila*: Integration of Positive and Negative Transcription Factor Inputs by a Minimal *eve* Enhancer

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ABSTRACT: We are interested in the mechanisms that generate neuronal diversity within the *Drosophila* central nervous system (CNS), and in particular in the development of a single identified motoneuron called RP2. Expression of the homeodomain transcription factor Even-skipped (Eve) is required for RP2 to establish proper connectivity with its muscle target. Here we investigate the mechanisms by which *eve* is specifically expressed within the RP2 motoneuron lineage. Within the NB4-2 lineage, expression of *eve* first occurs in the precursor of RP2, called GMC4-2a. We identify a small 500 base pair *eve* enhancer that mediates *eve* expression in GMC4-2a. We show that four different transcription factors (Prospero, Huckebein, Fushi tarazu, and Pdm1) are all expressed in GMC4-2a, and are required to activate *eve* via this minimal enhancer, and that one transcription factor (Klumpfuss) represses *eve* expression via this element. All four positively acting transcription factors act independently, regulating *eve* but not each other. Thus, the *eve* enhancer integrates multiple positive and negative transcription factor inputs to restrict *eve* expression to a single precursor cell (GMC4-2a) and its RP2 motoneuron progeny. © 2003 Wiley Periodicals, Inc. J Neurobiol 57: 193–203, 2003

Keywords: **motoneuron fate; *eve* regulation; positive and negative transcription**

INTRODUCTION

The generation of neuronal diversity is essential for assembling a functional central nervous system (CNS). The relatively simple development of the *Drosophila* embryonic CNS provides an ideal model system for studying how neuronal diversity is generated. The CNS develops from neural precursors, neuroblasts, which divide asymmetrically to generate a series of smaller ganglion mother cells (GMCs) (Doe and Skeath, 1996). GMCs are intermediate precursors that produce two postmitotic neurons or glia. Each of the 30 neuroblasts in a hemisegment can be uniquely identified based on its position, time of formation, and expression of molecular markers (Broadus et al., 1995); most importantly, each neuroblast generates a unique and reproducible clone of neurons and/or glia (Schmid et al., 1999).

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One of the most important processes in establishing neuronal diversity is the generation of distinct GMCs in each neuroblast lineage. Each GMC differs from other GMCs, even ones from the same neuroblast lineage; GMCs from the same neuroblast lineage form at different times, express different combinations of genes, and produce unique combinations of neurons or glia (Schmid et al., 1999; Isshiki et al., 2001). Relatively few genes are known that distinguish GMC identity. Recently, four "temporal identity" genes have been identified that play a common role in distinguishing early-born from later-born GMC fates in many neuroblast lineages. Neuroblasts sequentially express *hunchback* (*hb*), *Kruppel* (*Kr*), *pdm1*, and *castor* (*cas*), and GMCs born during each window of expression will maintain that pattern of gene expression in their neuronal progeny (Isshiki et al., 2001). In addition, *hb* and *Kr* are necessary and sufficient for first- and second-born GMC fate, respectively, in many neuroblast lineages (Isshiki et al., 2001). An open question, however, is how first-born GMC fates from different neuroblast lineages acquire their unique cell fates.

To investigate the specification of GMC identity at the level of a single, identified GMC, we and others have focused on the first-born GMC from the neuroblast 4-2 lineage, called GMC4-2a. GMC4-2a produces the Even-skipped (Eve) expressing RP2 motoneuron and a smaller sibling neuron that downregulates Eve expression (Doe, 1992). The mature RP2 neuron extends its axon ipsilaterally and synapses with dorsal muscle 2, a process that requires the activity of the Eve transcription factor (Doe et al., 1988b; Landgraf et al., 1999). A number of transcription factors are known to positively or negatively regulate expression of *eve* in GMC4-2a. Positive regulators include the homeoprospero domain (Ryter et al., 2002) protein Prospero (Pros) (Doe et al., 1991), the homeodomain protein Fushi tarazu (Ftz) (Doe et al., 1988a), the zinc finger protein Huckebein (Hkb) (Chu-LaGraff et al., 1995), and the functionally redundant Pou-type homeodomain proteins Pdm1 and Pdm-2 (also called Nubbin and Miti-mere, respectively; Yang et al., 1993; Bhat et al., 1995; Yeo et al., 1995). All of these nuclear proteins are detected in GMC4-2a, and mutations in any one of these genes results in loss of *eve* expression in GMC4-2a. Only one negative regulator is known: the nuclear zinc finger protein Klumpfuss (Klu) (Yang et al., 1997). Klu is detected in the second-born GMC in the lineage, GMC4-2b, but not in GMC4-2a; *klu* mutations produce duplicate RP2 motoneuron fates, probably due to a transformation of GMC4-2b into a GMC4-2a identity (Yang et al., 1997).

Each of the genes required to regulate *eve* expression in the NB4-2 lineage encode putative or known transcription factors with the potential to directly regulate *eve* expression or the genes expressed in GMC4-2a. This raises the question of whether these genes regulate each other, with only a subset directly regulating *eve* expression, or whether each gene is independently required to activate *eve* expression (without affecting expression of the other genes). It has been reported that Pros and Ftz activate expression of Pdm1 in GMC4-2a (Yang et al., 1993; Bhat et al., 1995), whereas Hkb is not required to activate any of the other genes (Chu-LaGraff et al., 1995). The relationship between other genes in this pathway has not been tested.

Here we explore the mechanism of *eve* regulation in GMC4-2a and its progeny, the RP2 motoneuron. We identify a 500 base pair minimal *eve* enhancer that mediates GMC4-2a and GMC4-2b gene expression in wild-type embryos. We show that this enhancer responds appropriately in mutants of positive and negative regulators, indicating that these transcription factors act through this enhancer to regulate *eve*. We show that all five transcription factors independently regulate *eve* expression, without affecting each other's expression. This indicates that the *eve* enhancer integrates multiple positive and negative transcription factor inputs to restrict expression to a single GMC within the NB4-2 lineage. Finally, we mutagenize three predicted Klu binding sites in the minimal *eve* enhancer and show that this alters the expression pattern of the *eve* reporter transgene in a manner consistent with blocking Klu function. This indicates that Klu is likely to directly bind to the *eve* enhancer and repress *eve* expression.

MATERIALS AND METHODS

Drosophila Strains

The *yellow white* strain was used for wild-type analysis. The following mutant lines were used: *ftzK6;ftz*^{9H34}/TM3 *ftz lacZ* (the *ftzK6* transgene partially rescues the early segmentation defects in *ftz* embryos; Doe et al. 1998a); *ftz*$^{Ual2RA9.1}$ (homozygous viable *ftz* allele; Kellerman et al., 1990); *hkb*^{XM9}/TM3 *ftz lacZ* (Bronner et al., 1994); *klu*^{P212IR51C}/*TM3 ftz lacZ* (Yang et al., 1997); *pros*^{14}/TM3 *ftz lacZ* (Doe et al., 1991); *Df(2L)GR4/CyO ftz lacZ* (deletion of both *pdm1* and *pdm-2*, but not *paired*; Yeo et al., 1995); *Scabrous-Gal4* (drives expression in neuroblasts and their progeny); *UAS-klumpfuss* (Yang et al., 1997). Standard genetic techniques were used to recombine the R79S84-*lacZ* transgene, an *eve-lacZ* insertion on the second chromosome driving expression in the RP2/aCC/pCC lineages (Fujioka et

al., 1999), with the mutant alleles listed above, except that the R79S84-*lacZ* transgene was recombined with only one *ftz* allele, $ftz^{Ual2RA9.1}$. All mutant strains used in this study are null alleles with the exception of $ftz^{Ual2RA9.1}$, which is wild-type for segmentation, but mutant for the NB 4-2 lineage; most RP2 neurons do not express Eve in $ftz^{Ual2RA9.1}$ embryos (J.A.M. data not shown; Kellerman et al., 1990).

Construction of Transgenic Lines and Site-Directed Mutagenesis

Standard techniques were used to construct the *eve-lacZ cis*-regulatory constructs and transgenic fly stocks (Rubin and Spradling, 1982; Fujioka et al., 2000). The subdivided neuronal elements were originally cloned downstream of a *lacZ* reporter (Fujioka et al., 1999). In this study, to obtain higher level expression for a more detailed analysis, the truncated elements [except C82R92 and S84R92, see Fig. 3(G) and Fujioka et al., 1999] were placed upstream of the *eve* DNA sequence from −275 to +166 bp (relative to the *eve* transcription start site, +1), fused to the *lacZ* coding sequence, followed by the *eve* 3′ UTR sequence from +1306 to +1521 bp, which was shown to increase the apparent translational efficiency (Fujioka et al., 1999).

Site-directed mutagenesis of the R79S84 *eve-lacZ* construct was performed using the Quick Change Site-Directed Mutagenesis kit (Stratagene) according to manufacturer's instructions. To mutate the three predicted Klu-binding sites (K123) we used the following oligonucleotides: for Klu-binding site 1 (+8066 to +8075) we used the forward primer CGAAAATCGAGGGAGAAGAGCGGAGGGGCG (where the underlined nucleotide is mutated from wild-type) and its complement; for Klu-binding site 2 (+8090 to +8099) we used the forward primer GGGGCGGGGTGCGAGAATGACTTGCCTGCC and its complement; for Klu-binding site 3 (+8262 to +8271) we used the forward primer CTGACACCATCGTTCTCCACCGCATCC and its complement. Subsequent R79S84 *eve*K123 mutagenized plasmids were isolated and sequenced to confirm that only the putative Klu binding sites were mutated. The mutagenized *eve*K123 fragments were cloned in the vector described above, and transgenic lines were obtained as described above.

Antibody Staining and Mutant Analysis

Embryo fixation, antibody staining, and detection of homozygous mutant embryos were performed as previously described (McDonald and Doe, 1997; McDonald et al., 1998). Embryos were staged according to Campos-Ortega and Hartenstein (1997). Primary antibodies were used at the following dilutions: rabbit anti-β-galactosidase serum (1:2000; Cappel); rat anti-β-galactosidase serum (1:5000; Srinivasan et al., 1997); mouse anti-Engrailed (4D9; 1:2; Patel et al., 1989); mouse anti-Eve monoclonal (2B8; 1:10; Patel et al., 1994); mouse anti-22C10 (1:10; Patel et al., 1994); rabbit anti-Eve serum (1:6000; Frasch et al., 1987); mouse anti-Ftz monoclonal (1:1200; Kellerman et al., 1990); rat anti-Gooseberry-distal monoclonal (16F12 and 10E10; 1:12; Zhang et al., 1994); rat anti-Hkb serum (1:100; McDonald and Doe, 1997); rat anti-Ind serum (1:75; Weiss et al. 1998); rabbit anti-Klu serum (1:500; Yang et al., 1997); rabbit anti-Pdm1 serum (1:100; Yeo et al., 1995); mouse anti-Pros monoclonal (MR2A; 1:4; Spana and Doe, 1995). We used secondary antibodies at a dilution of 1:400 conjugated to biotin (Vector Labs), alkaline phosphatase (Southern Biotechnology Associates), LRSC, DTAF, Cy5, streptavidin-Cy5 (Jackson Immunoresearch), Alexa 488, and Alexa 594 (Molecular Probes).

For histochemical analysis, embryos mounted in 85% glycerol were viewed on a Zeiss Axioplan microscope and images were acquired with a Sony DKC-5000 digital camera. Fluorescently stained embryos were mounted in 85% glycerol with 1% N-propylgallate and viewed on a Biorad confocal microscope. In some experiments, the primary antibody was detected using a secondary antibody conjugated to biotin in conjunction with the Vectastain Elite kit (Vector Labs) and the Renaissance TSA Indirect kit (NEN) to amplify the signal. Figures were assembled in Adobe Photoshop.

RESULTS

Timing of Transcription Factor Expression in GMC4-2a and GMC4-2b

GMC4-2a forms at stage 9, becomes Eve^+ at stage 11, and generates the Eve^+ RP2/sib neurons at late stage 11 (Doe, 1992; Yang et al., 1997). The second-born Eve-negative GMC4-2b forms at stage 10, and generates an unknown pair of neurons (Yang et al., 1997). The first transcription factors detected in GMC4-2a are Pros and Hkb [Fig. 1(A) and (C); Table 1], due to inheritance of the proteins from the neuroblast. The next transcription factors detected in GMC4-2a are Ftz and Pdm1. Ftz is first detected at stage 10 [Fig. 1(B)], and Pdm1 is first detected at stage 11 [Fig. 1(D)]. The *de novo* expression of Pdm1 is distinct from its inheritance in GMCs produced by $Pdm1^+$ neuroblasts during the assignment of temporal identity (Isshiki et al., 2001). The last protein to be detected is Eve, which appears only at late stage 11 [Fig. 1(C), (H), and (I)]. Pros, Hkb, Ftz, and Pdm1 are each expressed transiently in the RP2/sib neurons at stage 12, but by stage 16 none of these proteins is detectable in the mature RP2 neuron [Fig. 1(I); J.A. McDonald and C.Q. Doe, unpublished results]. We conclude that there is a temporal sequence of transcription factor expression in GMC4-2a: first Pros and Hkb, then Ftz, then Pdm1, and that Eve is detected only after all of these proteins are present.

GMC4-2b forms at late stage 10, never expresses

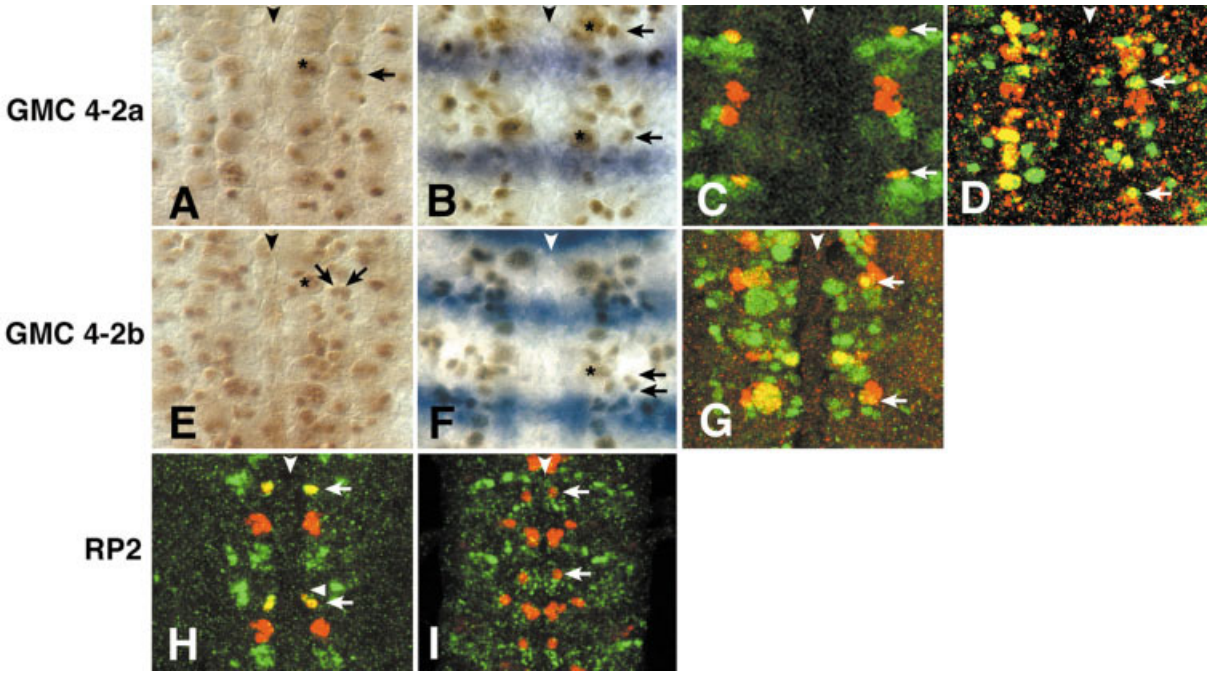

Figure 1

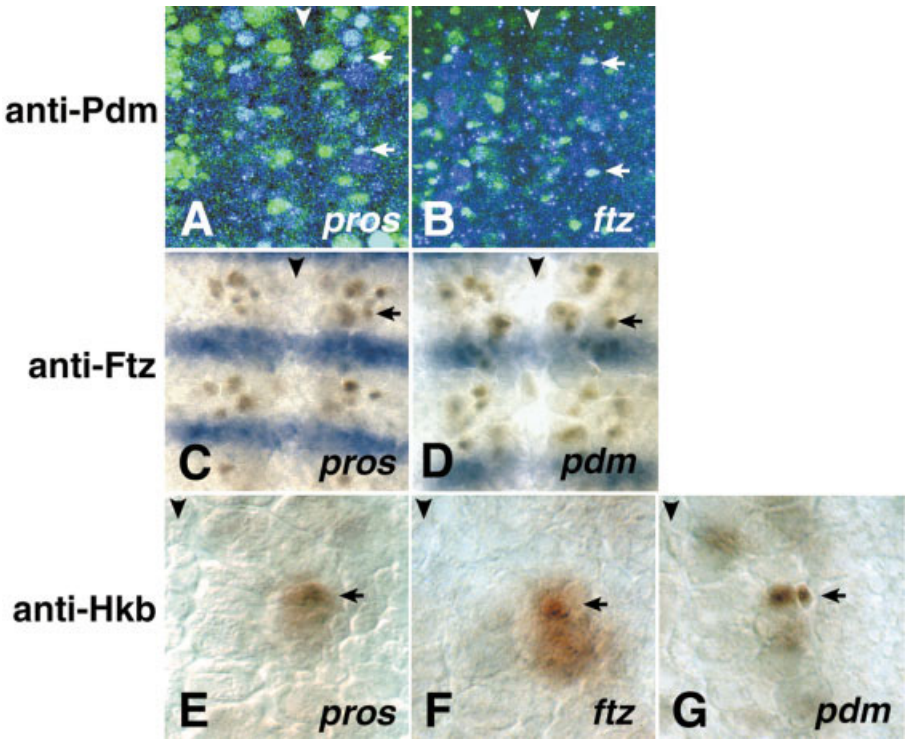

Figure 2 Transcription factor regulation in the NB4-2 lineage. (A–B) Pdm expression in stage 11 *pros* (A) and *ftz* (B) embryos. Pdm (green) and Ind (blue) are coexpressed (cyan) in GMC4-2a (arrow). (C–D) Ftz expression in stage 10 *pros* (C) and *pdm* (D) embryos. The Ftz^{+} GMC4-2a (brown; arrow) is adjacent to NB4-2 and anterior to row 5 Gsb expression (purple stripes). (E–G) Hkb expression in stage 10 *pros* (E), *ftz* (F), and *pdm* (G) embryos. The Hkb^{+} GMC4-2a (arrow) is adjacent to NB4-2. In all panels, anterior is up, the ventral midline is marked with an arrowhead, and a ventral view is shown. Two segments are shown for (A–D), and one segment for (E–G).

Figure 1 Transcription factor expression in the NB4-2 lineage. Wild-type embryos showing expression of Pros (A,E), Ftz (B,F; brown), Hkb (C,G,H,I), Pdm1 (D), and Klu (G) in the following cells of the NB4-2 lineage: GMC4-2a (A–D), GMC4-2b (E–G), and RP2 neuron (H,I). Two segments are shown; anterior, up; ventral midline, arrowhead. (A–D) Transcription factors in GMC4-2a (arrow). (A) Pros is detected at stage 9; GMC4-2a is identified by its position lateral to the Pros-expressing MP2 neuroblast (asterisk). (B) Ftz (brown) is detected at stage 10; GMC4-2a is identified by its position lateral to the Ftz-expressing MP2 neuroblast (asterisk) and anterior to row 5 neuroblasts expressing Gooseberry (purple stripes). (C) Hkb (green) is detected at stage 11 (it is first detectable at stage 9; not shown); GMC4-2a is identified as a single Eve^+ GMC (red; coexpression makes it appear yellow) between the clusters of Eve^+ progeny from NBs 1-1 and 7-1. (D) Pdm1 (green) is detected at stage 11; GMC4-2a is identified by Ind (red; coexpression makes it appear yellow), a marker for NB 4-2 and GMC4-2a, and by its position relative to the row 6 Engrailed-expressing neuroblasts (data not shown). (E–G) Transcription factors in GMC4-2b (arrow). (E) Pros is detected in both GMC4-2a (arrow) and GMC4-2b at late stage 10; GMC4-2b is identified as one of two GMCs closely associated with NB4-2. (F) Ftz is detected in both GMC4-2a (arrow) and GMC4-2b at late stage 10; GMC4-2b is identified as one of two GMCs closely associated with NB4-2. (G) Klu (green) and Hkb (red) are detected in GMC4-2b at stage 11 (coexpression makes the GMC appear yellow); two GMCs are closely associated with NB4-2, GMC4-2a is Eve^+ (blue; not shown) and GMC4-2b is Eve-negative. (H–I) Hkb in the RP2 motoneuron (arrow). (H) At stage 12 Hkb (green) is detected in the Eve^+ (red) new-born RP2 and RP2sib neurons; coexpression makes it appear yellow. (I) at stage 16 Hkb (green) is no longer detected in the Eve^+ (red) mature RP2 motoneuron.

Eve, and generates two unknown Eve-negative neurons (Yang et al., 1997). Three transcription factors that positively regulate *eve* expression are detected in GMC4-2b: Pros, Ftz, and Hkb [Fig. 1(E)–(G)]. The pattern of Pdm1 expression is too complex to score at the time GMC4-2b is born (late stage 11). We confirm the previous observation (Yang et al., 1997) that the negative regulator Klu is detected in GMC4-2b [Fig. 1(G)] but not GMC4-2a (data not shown). We conclude that GMC4-2b expresses at least three of the four positively acting transcription factors that are required to activate *eve* (Pros, Ftz, Hkb), and at least one negative regulator of *eve* expression (Klu). The absence of *eve* expression is likely due to the presence of Klu, rather than the absence of a positive regulator, because *klu* mutants can activate *eve* transcription in GMC4-2b (Yang et al., 1997).

Table 1 Expression of Markers in the NB4-2 Lineage

Marker	GMC4-2a	RP2/sib	GMC4-2b
Eve	+	+	−
Eve-lacZ[b]	+	+	−
Ftz	+	+[a]	+
Hkb	+	+[a]	+
Ind	+	ND[c]	+
Pdm	+	+[a]	ND
Pros	+	+[a]	+
Klu	−	−	+

[a] Ftz, Hkb, Pdm, and Pros are transiently expressed in RP2 and RP2sib at stage 12 but are not detected by stage 13 (Doe et al., 1988a; Bhat et al., 1995; and data not shown).

[b] Eve-lacZ is the 0.5 kb R79S84 element.

[c] (ND) Not determined.

Pros, Hkb, Ftz, and Pdm1 Regulation of *eve* Expression in GMC4-2a

The sequential expression of Pros, Hkb, Ftz, Pdm1, and Eve in GMC4-2a raises the possibility that these four transcription factors act in a linear pathway to regulate *eve* expression. If so, then a mutant in an early-acting gene should lead to loss of expression of all later-acting genes in the pathway. Alternatively, the four transcription factors could all act directly to activate *eve* transcription, with expression of *eve* occurring only after all transcription factors are present. In this case, mutants in one gene should have no effect on any other gene except *eve*. To distinguish between these two models, we examined *pros*, *hkb*, *ftz*, and *pdm1* mutants for expression of all four transcription factors and *eve* (Fig. 2, Table 2). We find that Pdm1 is detected in GMC4-2a in all mutant genotypes: *pros* [Fig. 2(A)], *ftz* [Fig. 2(B)], and *hkb* (Chu-LaGraff et al., 1995); Ftz is detected in GMC4-2a in all mutant genotypes: *pros* [Fig. 2(C)], *hkb* (Chu-LaGraff et al., 1995), and *pdm1* [Fig. 2(D)]; and Hkb is detected in GMC4-2a in all mutant genotypes [Fig. 2(E)–(G)]. Finally, we observe Pros in GMC4-2a in all mutant genotypes (data not shown), as expected because Pros is transcribed and translated in neuroblasts and is

asymmetrically partitioned into each GMC (Spana and Doe, 1995; Broadus et al., 1998). Taken together, our data support the model that all four transcription factors act directly to activate *eve* transcription, with expression of *eve* occurring only after all transcription factors are present.

Definition of a Minimal *eve cis*-Regulatory Element for the NB4-2 Lineage

To test the model that Pros, Hkb, Ftz, and Pdm1 transcription factors directly regulate *eve* expression, we needed to identify the *eve cis*-regulatory DNA that confers regulated expression in the NB4-2 lineage. Eve is expressed in a subset of neurons in the embryonic CNS, including the aCC/pCC neurons derived from NB1-1, the U1-5 neurons derived from NB7-1, the EL neurons derived from NB 3-3, and the RP2/sib neurons derived from NB4-2 (Doe, 1992; Higashijima et al., 1996; Isshiki et al., 2001). Fujioka et al. (1999) previously defined an *eve cis*-regulatory element (R79R92; from +7.9 and +9.2 kilobase pair (kb) on the *eve* genomic map) that accurately directs *lacZ* expression to the Eve^+ cells within two NB lineages: GMC4-2a and its RP2 progeny and GMC1-1a and its aCC/pCC progeny. Here we examined the properties of this element in detail. When the R79R92 *eve* element was truncated to +7.9 to +8.6 kb (R79N86), *lacZ* expression in RP2 and aCC was normal, whereas expression in the pCC neuron was reduced (Fig. 3G). Truncation of the *eve* element to +7.9 to +8.4 kb (R79S84) almost completely abolished expression of *lacZ* in pCC, although occasionally expression in pCC was observed at low levels (data not shown), whereas expression in RP2 and aCC remained high [Fig. 3(A) and (G)]. Further truncation of the left end point to +8.0 kb [S80S84, Fig. 3(G)] resulted in a reduction of expression in both aCC and RP2. Addition of the region +8.4 to +8.6 kb to this fragment (S80N86) increased the level of expression. However, because the region +8.4 to + 9.2 kb (S84R92) did not show any ability to activate *lacZ*, the region +8.4 to +8.6 kb is apparently insufficient on its own to direct expression, and thus serves an auxiliary function. The removal of +8.2 to +8.4 kb from P80N86 abolished expression [SNΔSC in Fig. 3(G)]. Together with the fact that each of the fragments +7.9 to +8.2 kb (S79C82) and +8.2 to +9.2 kb (C82R92) failed to activate *lacZ* [Fig. 3(G)], this indicates that both of the regions +7.9 to +8.2 kb and +8.2 to +8.4 kb are necessary to direct expression, and that neither alone is sufficient. Consistent with this, two tandem copies of +8.2 to +8.4 kb failed to activate *lacZ* (C82S84x2), suggesting that the two regions may provide qualitatively different activities. In summary, the critical *eve cis*-regulatory element for the GMC4-2a and RP2 lies in a 0.5 kb fragment of genomic DNA between +7.9 and +8.4 kb [Fig. 3(G); (Tables 1 and 2)].

We wanted to know whether the genes that activate or repress *eve* expression in the NB4-2 lineage work through the minimal 500 bp RP2/aCC *eve* enhancer. We assayed expression of R79S84-*lacZ* in *pros*, *ftz*, *hkb*, *pdm1*, and *klu* mutant embryos (Fig. 3; Table 2), and tested whether it was regulated identically to the endogenous *eve* gene. We find that *ftz* [Fig. 3(B)], *pdm1* [Fig. 3(C)], and *hkb* [Fig. 3(D)] mutant embryos show loss of R79S84-*lacZ* in the RP2 neuron but not the aCC neuron, identical to the pattern of endogenous *eve* expression in these mutants (Doe et al., 1988a; Yang et al., 1993; Chu-LaGraff et al., 1995; Yeo et al., 1995). We find that *pros* mutants show loss of *eve-lacZ* in both RP2 and aCC [Fig. 3(E)], identical to the pattern of endogenous *eve* expression in pros mutants (Doe et al., 1991). In embryos lacking *klu*, R79S84-*lacZ* is expressed in two cells at the RP2 position, whereas expression in aCC is normal [Fig. 3(F)]; this matches the pattern of endogenous *eve* expression in *klu* mutant embryos (Yang et al., 1997). We conclude that the R79S84 minimal *eve cis*-regulatory element precisely reproduces the pattern of endogenous *eve* expression within the NB4-2 lineage, and that transcription factors regulating *eve* in GMC4-2a can act through this enhancer to activate or repress *eve* expression.

Repression of *eve* Expression in GMC4-2b

Expression of *eve* is not detected in GMC4-2b in wild-type embryos, but mutations in the *klu* gene result in ectopic expression of *eve* in GMC4-2b. Klu contains four predicted zinc fingers, one of which is highly homologous to the WT1 zinc finger domain (Klein and Campos-Ortega, 1997). The consensus binding site for the WT1 zinc finger transcription factor is a ten nucleotide sequence, 5′-(C/G/T)CGT-GGG(A/T)(G/T)(T/G)-3′, with variable nucleotides shown in parentheses (Lee and Pelletier, 2001). We reasoned that if Klu directly binds to the *eve* enhancer to repress expression in GMC4-2b, one or more WT1 consensus binding sites should be found in the minimal *eve* enhancer R79S84. We found three conserved putative Klu-binding sites in the R79S84 sequence: site 1, GGGTGGGGAG at nucleotides +8066 to +8075; site 2, GCGTGGGTGA at nucleotides +8090 to +8099; and site 3, TCGCCCACCA at

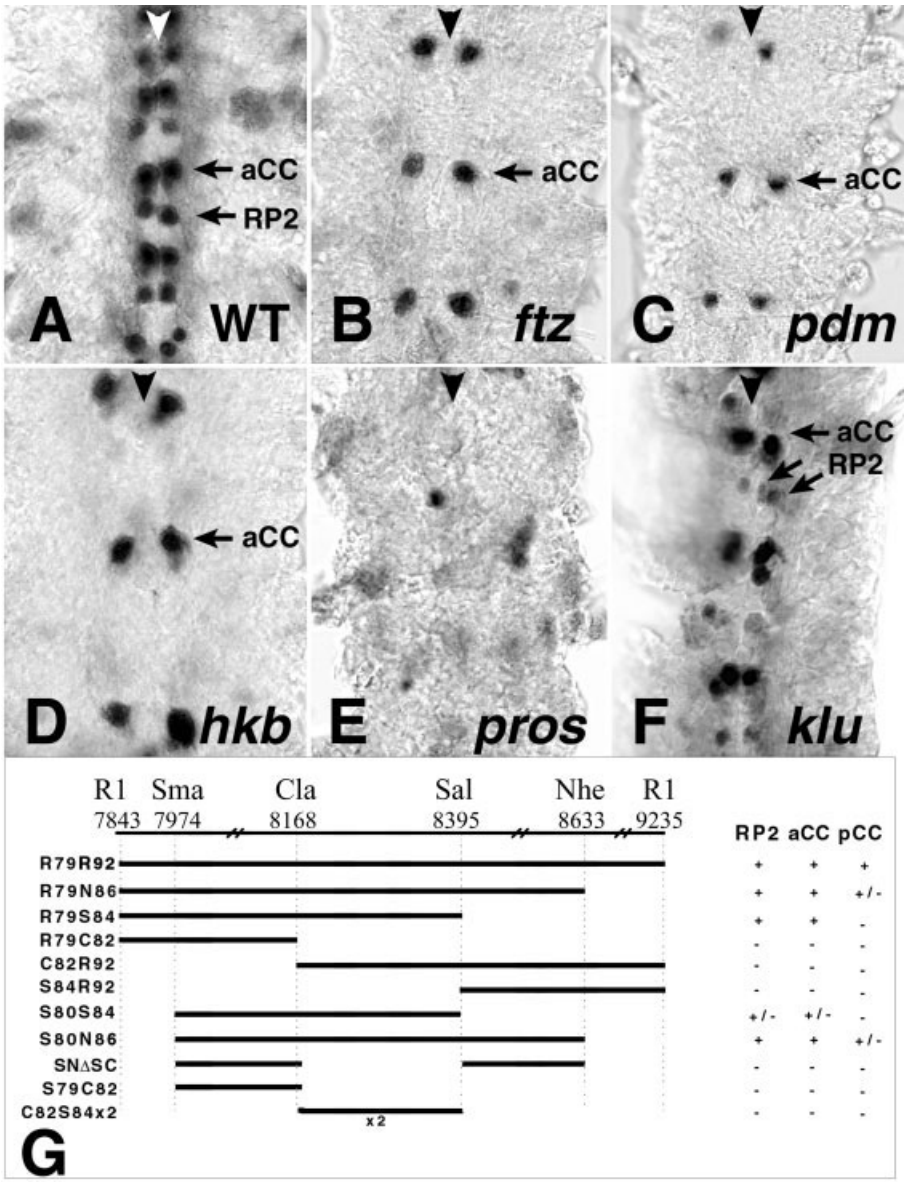

Figure 3 Definition of a minimal *eve cis*-regulatory element for the NB 4-2 lineage. (A–F) Dorsal view of two segments of stage 15 and 16 dissected nerve cords showing the pattern of R79S84-*lacZ* expression in wild-type (A), *ftz* (B), *pdm* (C), *hkb* (D), *pros* (E), and *klu* (F) embryos. The Eve^{+} RP2 and aCC neurons are labeled (arrows). (G) Diagram of the *eve* enhancer region. The top line is the original enhancer region; the restriction enzyme sites are shown (RI, EcoRI; Cla, ClaI; Sal, SalI; Nhe, NheI); the nucleotide position, in base pairs downstream of the Eve transcription start site, is indicated above the line. The transgenes tested are named based on the base pair position and the restriction sites used to construct the line; the extent of the construct is shown as a line below the enhancer region. The level of *lacZ* transgene expression in RP2, aCC, or pCC is indicated next to each transgene by a + (expressed), − (not expressed), and +/− (weakly expressed). In (A–F), anterior is up and the midline is indicated by an arrowhead.

+8262 to +8271 [Fig. 4(A)]. Based on the fact that altering the C_2, G_3, G_5, G_6, and G_7 to T or T_4 to A in the WT1-consensus binding site abolished WT1 binding (Lee and Pelletier, 2001), we made nucleotide substitutions in the three putative Klu-binding sites. In sites 1 and 2, we substituted an A for T_4, G_6, and G_7 [Fig. 4(A)]. In site 3, which is a reversed binding site, we substituted a T for C_4, C_6, and A_7 [Fig. 4(A)]. We made these substitutions at all three sites, constructed transgenic lines expressing the mutant enhancer driv-

Table 2 Summary of Results

Marker	*WT*[a]	*ftz*$^-$	*hkb*$^-$	*pdm*$^-$	*pros*$^-$	*klu*$^-$
Eve-*lacZ*+ RP2	94%[b] (102)	11% (146)[c]	0% (96)	0% (112)	0% (74)	45% (80)[d]
Ftz+ GMC4-2a	86% (70)	NA	100%[e]	92% (48)	95% (86)	ND
Hkb+ GMC4-2a	94% (150)	84% (51)	NA	80% (67)	91% (44)	ND
Pdm+ GMC4-2a	100% (34)	100% (51)	100%[e]	NA	94% (34)	ND
Pros+ GMC4-2a	95% (94)	99% (81)	100%[e]	96% (96)	NA	ND

[a] *Abbreviations:* WT, wildtype; NA, not applicable; ND, not determined; Eve-lacZ, the 0.5 kb R79S84-*lacZ* transgene.

[b] The % of hemisegments with cells expressing the marker; the number in parentheses is the number of hemisegments examined for each genotype; all hemisegments were assayed, even those in which a GMC could not be detected.

[c] For analysis of Eve-*lacZ* expression, the *ftz*$^{UAL2RA9.1}$ allele was used, which exhibits a small percentage of Eve+ neurons (5%, $n = 168$ h.s.); all other phenotypic analyses were done with the *ftzK6;ftz*^{9H34} allele.

[d] Number of duplicated RP2 neurons per hemisegment in *klu* embryos; for T2-A1 segments, 83% of hemisegments have duplicated RP2 neurons; for A2–A8, 29% of hemisegments have duplicated RP2 neurons.

[e] Chu-LaGraff et al. (1995).

ing *lacZ* (*eve*K123-*lacZ*; Fig. 4), and examined the pattern of *lacZ* expression in the CNS of wild-type embryos and embryos misexpressing a Klu protein in the NB4-2 lineage.

In wild-type embryos, the *eve*K123-*lacZ* transgene is expressed in the aCC and RP2 neurons, similar to the wild-type (R79S84) *eve-lacZ* transgene. However, in one or two hemisegments per embryo, we observed an extra cell expressing *eve*K123-*lacZ* adjacent to the RP2 neuron [Fig. 4(B); $n = 5$ embryos]. This phenotype is very similar to wild-type (R79S84) *eve-lacZ* expression in *klu* mutant embryos [compare to Fig. 3(F)], although slightly less penetrant. We conclude that the *eve*K123-*lacZ* transgene mimics the *klu* mutant phenotype, and propose that Klu represses *eve* expression via direct binding to one or more of these sites.

To further test this hypothesis, we used gain of function experiments to test whether ectopic Klu in GMC4-2a can repress *eve-lacZ* expression via these sites. We compared expression of a wild-type (R79S84) *eve-lacZ* transgene with a transgene containing three mutated Klu consensus binding sites (*eve*K123-*lacZ*) in embryos where *Scabrous-Gal4* (*Sca-Gal4*) drives ectopic expression of *UAS-klu* in all neuroblast lineages [Fig. 4(C)–(D)]. We find that the wild-type (R79S84) *eve-lacZ* expression is partially repressed by ectopic Klu expression [Fig. 4(C); 0 positive RP2 neurons, $n = 5$ embryos], but that the *eve*K123-*lacZ* transgene with mutated Klu sites is repressed to a lesser extent [Fig. 4(D); 2 positive RP2 neurons, $n = 5$ embryos]. This difference in repression is only observed when the levels of transgene expression are lowered by raising the embryos at 18°C; when the transgenes are more strongly expressed (by raising the embryos at 23°C) we observe no detectable repression (data not shown). Taken together, our Klu loss of function and misexpression studies indicate that Klu acts partly, but not completely, through three predicted Klu-binding sites to repress *eve* expression in the NB4-2 lineage.

DISCUSSION

In contrast to the models previously proposed (Chu-LaGraff et al., 1995; Yeo et al., 1995), we find that *hkb*, *ftz*, *pdm1*, and *pros* are independently required to activate *eve* expression in GMC4-2a. This suggests that the *eve* enhancer is capable of integrating the input of all four of these transcription factors to activate transcription. Recently, we have also determined that Hb and Ind are also necessary for *eve* expression in GMC4-2a (Weiss et al., 1998; Isshiki et al., 2001), but we do not know if they act directly on the *eve* element or via one of the four transcription factors described here. We found putative binding sites for each of the positively acting transcription factors within the minimal *eve* element, but mutation of these sites had no effect on expression of the *eve-lacZ* transgene in embryos (M. Fujioka, J.A. McDonald, and C.Q. Doe, unpublished results). It remains to be determined whether Pros, Hkb, Ftz, or Pdm1 activate *eve* transcription via direct binding to the minimal *eve* element, or indirectly by activating or facilitating the binding of other transcriptional activators.

Based on functional dissection of the RP2/aCC/pCC *eve* element, it seems to be composed of three parts. The regions +7.9 to +8.2 kb and +8.2 to +8.4 kb are each necessary to direct the expression pattern (together they comprise the minimal element for expression in RP2 and aCC), while the region +8.4 to +8.6 kb enhances the level of expression. Expression in the pCC neuron is further enhanced by the region extending to +9.2 kb. The two regions within the minimal element seem to be regulated by different

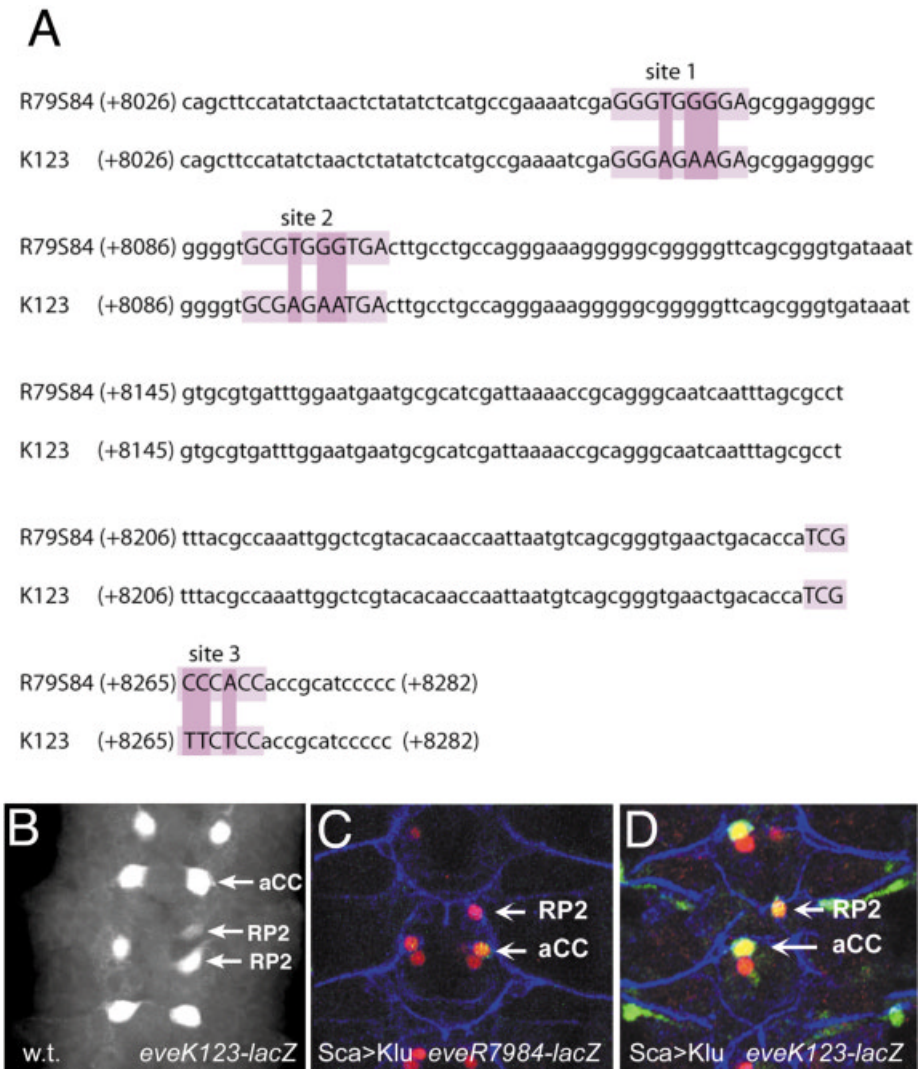

Figure 4 Mutating putative Klu-binding sites alters the *eve-lacZ* expression pattern. (A) A portion of the R79S84 *eve* enhancer sequence, from nucleotides +8026 to +8282, showing three putative Klu-binding sites (light purple and capitalized): site 1, GGGTGGGGAG at nucleotides +8066 to +8075; site 2, GCGTGGGTGA at nucleotides +8090 to +8099; and site 3, TCGCCCACCA at +8262 to +8271. Top line, wild-type sequence; bottom line, sequence of the element with all three predicted Klu-binding sites mutated (*eve*K123; nucleotide substitutions in dark purple). (B) Wild-type embryo expressing a transgene containing three mutated Klu consensus binding sites (*eve*K123-*lacZ*). There is a low frequency of ectopic *eve-lacZ* expression in the RP2 neuron (arrow), which is never observed in embryos containing the wild-type *eve-lacZ* transgene [compare to Fig. 3(A)]. (C–D) Mutating three Klu consensus binding sites partially antagonizes Klu misexpression phenotypes. Embryos stained for endogenous Eve (red), β-gal (green, to monitor transgene expression), and 22C10 (blue, as a landmark for RP2 position). Anterior, up; ventral midline, panel center. (C) A wild-type (R79S84) *eve-lacZ* transgene is strongly repressed by *Sca-Gal4; UAS-klu* misexpression at 18°C. (D) A transgene containing three mutated Klu binding sites (*eve*K123-*lacZ*) shows slightly less repression by *Sca-Gal4; UAS-klu* misexpression at 18°C (note the positive RP2 neuron).

factors, because two copies of +8.2 to +8.4 kb (increasing the number of activator binding sites within this region by twofold) could not substitute for the function of the region +7.9 to +8.2 kb. This is consistent with the fact that at least four factors are independently required to activate *eve* in RP2 neurons.

How does Klu repress *eve* expression in GMC4-

2b? We have evidence that negative regulation of *eve* expression by Klu is due to direct binding to the *eve* minimal element. First, we show that *klu* mutants exhibit similar derepression of the *eve* minimal element transgene and the endogenous *eve* gene in the NB4-2 lineage; second, we detect three consensus binding sites for Klu in the *eve* minimal element (comparison of *Drosophila virilis* and *Drosophila melanogaster* shows that the three identified sites are highly conserved); third, mutation of these sites results in ectopic expression of *eve-lacZ* in the NB4-2 lineage in wild-type; and fourth, mutation of these sites impairs repression of *eve-lacZ* by ectopic Klu in the NB4-2 lineage. The predicted Klu binding sites (K123) are probably only a subset of relevant Klu binding sites, however, because mutation of the sites gives only partially penetrant phenotypes.

Surprisingly, we could not separate the GMC4-2a/RP2 element from the GMC1-1a/aCC/pCC element. In both NB 1-1 and NB 4-2 lineages, *eve* is expressed in the first-born GMC and its neuronal progeny. Both first-born GMCs share expression of several transcription factors, including Pros and Ftz. However, many other transcription factors are differentially expressed, such as the GMC1-1a specific expression of Vnd and Odd-skipped, and the GMC4-2a specific expression of Hkb, Pdm1, and Ind. It is possible that one or more commonly expressed transcription factors are required for expression of *eve* in both GMC1-1a and GMC4-2a, such as Pros, and this is why the elements cannot be subdivided.

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