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.

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).
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 (Schmidt et al., 1999; Ishhiki 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*), *Krüppel* (*Kr*), *pdm1*, and *castor* (*cas*), and GMCs born during each window of expression will maintain that pattern of gene expression in their neuronal progeny (Ishhiki et al., 2001). In addition, *hb* and *Kr* are necessary and sufficient for first- and second-born GMC fate, respectively, in many neuroblast lineages (Ishhiki 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 each other, with only a subset directly regulating 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: *ftz*K6;*ftz*ΔH13/TM3 *ftz* lacZ (the *ftz*K6 transgene partially rescues the early segmentation defects in *ftz* embryos; Doe et al. 1998a); *ftz*Δ12RA0.1 (homozygous viable *ftz* allele; Kellerman et al., 1990); hkb149/+;TM3 *ftz* lacZ (Bronner et al., 1994); klu21211R51C/TM3 *ftz* lacZ (Yang et al., 1997); *pros*Δ4/TM3 *ftz* lacZ (Doe et al., 1991); Df(2L)GR4/CyO *ftz* lacZ (deletion of both *pdm1* and *pdm2*, but not *paired*; Yeo et al., 1995); Scebrass-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
with the mutant alleles listed above, except that the R79S84-lacZ transgene was recombined with only one ftz allele, ftz13a2ras1. All mutant strains used in this study are null alleles with the exception of ftz13a2ras1, which is wild-type for segmentation, but mutant for the NB 4-2 lineage; most RP2 neurons do not express Eve in ftz13a2ras1 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 (Fujio 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 CGAAAATCGAGGGAGAAAGCGGAGGGCC (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 GGGCGGGGTGC-GAGAATGACTTGCCTGCC and its complement; for Klu-binding site 3 (+8262 to +8271) we used the forward primer CTGACACCATGTTCTCCACCGCATCC and its complement. Subsequent R79S84 eveK123 mutagenized plasmids were isolated and sequenced to confirm that only the putative Klu binding sites were mutated. The mutagenized eveK123 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
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 1-7-1. (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

<table>
<thead>
<tr>
<th>Marker</th>
<th>GMC4-2a</th>
<th>RP2/sib</th>
<th>GMC4-2b</th>
</tr>
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<tbody>
<tr>
<td>Eve</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Eve-lacZ</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Ftz</td>
<td>+</td>
<td>+(^a)</td>
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<tr>
<td>Hkb</td>
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<td>Ind</td>
<td>+</td>
<td>ND(^a)</td>
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<tr>
<td>Pdm</td>
<td>+</td>
<td>+(^a)</td>
<td>ND</td>
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<tr>
<td>Pros</td>
<td>+</td>
<td>+(^a)</td>
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<tr>
<td>Klu</td>
<td>−</td>
<td>−</td>
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</table>

\(^a\) 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; Ishihiki 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. 3A 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 [SnADC 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, pdml, 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)], pdml [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 observed 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’-CGTGGG(T/A)(G/T)/(C/T)-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, GGGTGGGAG at nucleotides +8066 to +8075; site 2, GGGTGGGTGA at nucleotides +8090 to +8099; and site 3, TGGCCACCA at
Based on the fact that altering the C2, G3, G5, G6, and G7 to T4 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 T4, G6, and G7 [Fig. 4(A)]. In site 3, which is a reversed binding site, we substituted a T for C4, C6, and A7 [Fig. 4(A)]. We made these substitutions at all three sites, constructed transgenic lines expressing the mutant enhancer driv-
expression in 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 eveK123-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 eveK123-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 (eveK123-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 eveK123-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
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 re-
shows that the three identified sites are melanogaster (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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