Expression and Function of the Segmentation Gene *fushi tarazu* During *Drosophila* Neurogenesis

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Segmentation genes control cell identities during early pattern formation in *Drosophila*. One of these genes, *fushi tarazu* (*ftz*), is now shown also to control cell fate during neurogenesis. Early in development, *ftz* is expressed in a striped pattern at the blastoderm stage. Later, it is transiently expressed in a specific subset of neuronal precursor cells, neurons (such as aCC, pCC, RP1, and RP2), and glia in the developing central nervous system (CNS). The function of *ftz* in the CNS was determined by creating *ftz* mutant embryos that express *ftz* in the blastoderm stripes but not in the CNS. In the absence of *ftz* CNS expression, some neurons appear normal (for example, the aCC, pCC, and RP1), whereas the RP2 neuron extends its growth cone along an abnormal pathway, mimicking its sibling (RP1), suggesting a transformation in neuronal identity.

DURING INSECT NEUROGENESIS, AN UNDIFFERENTIATED sheet of ventral ectoderm is transformed into a highly differentiated central nervous system (CNS). Each hemisegment contains about 250 neurons, many of which can be individually identified according to their characteristic morphology and synaptic connectivity. The generation of neuronal specificity occurs in two steps. First, in each segment, the morphologically uniform ventral ectoderm differentiates into a stereotyped array of neuronal precursor cells, namely, neuroblasts (NB's) and midline precursors (MP's), and nonneuronal cells. Second, each NB within this array divides asymmetrically to generate a characteristic chain of progeny, called ganglion mother cells (GMC's), each of which divides once more symmetrically to produce pairs of sibling neurons (*1, 2*). Unlike NB's, the MP's divide only once to generate two neuronal progeny (*3*). Each of the NB's and MP's can be individually identified according to its position within the array, and more significantly, according to the identified neurons it generates (*4*).

Experiments with laser ablations (*5, 6*) show that any cell in the neuroectoderm can become an NB, but once a cell enlarges to become an NB, it inhibits its neighbors from doing so and causes them to differentiate into nonneuronal cells. Each NB appears to be assigned its individual identity according to the position at which it enlarges within the neuroectoderm. Determined by its position, each NB goes on to generate its characteristic family of GMC's by an invariant cell lineage. Thus the fate of each individual neuron is determined both by its lineage and interactions with other neurons.

This model for insect neurogenesis leads to the prediction of several different classes of genes involved in the control of cell fates during neurogenesis. In *Drosophila*, "neurogenic" genes such as *Notch* appear to be involved in the local interactions controlling the decision of cells to become either NB's or nonneuronal cells (*7, 8*). The regulatory genes involved in the positional determination of individual neuronal precursor cells and the lineal determination of their progeny have yet to be identified.

In contrast, much is already known about the regulatory genes that control the earlier events of pattern formation involved in segmentation and segment identity. Two classes of genes that are required zygotically to establish the normal pattern of segmentation have been identified: segmentation (gap, pair-rule, and segment polarity) genes, which establish segment number and polarity (*9*); and homeotic genes, which control segment identity (*10*).

One of the best studied segmentation genes is *fushi tarazu* (*ftz*) (*11–13*). Loss of *ftz* function produces an embryonic lethal "pair rule" phenotype. The *ftz* gene contains a homeobox (*11, 12*) and is thus likely to encode a DNA binding protein (*12, 14*). The *ftz* protein is localized in the nucleus (*15*), consistent with its role as a regulatory protein. The *ftz* transcript and protein are localized in seven stripes at the cellular blastoderm stage at a double segment periodicity (*15, 16*); these stripes are coincident with the primordia of the deleted pattern elements in the *ftz* mutant embryos (*13, 15–17*).

A surprising finding from localization studies with *ftz* antibodies (*15*) and the expression of *ftz/lacZ* fusion genes (containing the *ftz* promoter and the *lacZ* coding region) (*18*) is that *ftz* is transiently expressed a second time during embryogenesis in a segmentally repeated pattern in the developing CNS. Given the role that this regulatory gene plays in cell determination at the blastoderm stage, we wondered whether *ftz* might also be involved in cell determination during neurogenesis.

To test the potential role of *ftz* during neurogenesis, we first analyzed the pattern of *ftz* expression in the developing CNS, focusing our attention on several specific neuronal lineages that contain *ftz*-expressing neurons (herein called *ftz*$^+$ neurons). We then created *ftz* mutant flies that direct normal *ftz* expression in the blastoderm stripes, but not in the developing CNS. The phenotype of these mutant flies suggests that *ftz* controls cell identity during neurogenesis.

Transient expression of *ftz* in a segmentally repeated subset of neuronal precursor cells, neurons, and glia. We monitored the spatial and temporal pattern of *ftz* expression with antisera to assay

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for *ftz* protein (*15, 19, 20*), or by assaying for β-galactosidase (β-gal) in transformant (also called transgenic) embryos carrying a *ftz/lacZ* fusion gene (Fig. 1) (*18, 20, 21*). Both methods show the same spatial pattern of *ftz* CNS expression (with one minor exception; see Fig. 2). The advantage of the first method is that it shows the temporal expression pattern of *ftz* protein. The β-gal method, however, allows for easier identification of the ftz^+ neurons since β-gal persists longer than the native *ftz* protein, presumably due to the stability of the transcript or protein (or both), and thus is present in ftz^+ neurons after axonogenesis has begun (by this stage, the *ftz* protein has largely disappeared). In addition, β-gal is localized in the cytoplasm rather than the nucleus and reveals the structure of the cells including their axons, thus permitting us to unequivocally identify the neurons.

By the time of germ band elongation, the initial blastoderm striped expression of the *ftz* protein has largely disappeared. After about 1 hour, during which no *ftz* protein is detectable (4 to 5 hours), *ftz* expression in the developing CNS begins in a segmentally repeated subset of neuronal precursor cells. Of the approximately 250 neurons generated in each hemisegment, *ftz* is expressed in about 30 nuclei per hemisegment at hour 8 of development, and by hour 12 it is no longer expressed in the CNS.

Midline precursor lineages. Three MP's, a bilateral pair of MP2's and the unpaired MP1 along the midline, have been identified in the *Drosophila* embryo (*3, 22*). The MP1 and both MP2's express *ftz*, as do their pairs of neuronal progeny. MP2 is the first neuronal cell to express *ftz*; it begins to express *ftz* as it delaminates from the ectoderm into the NB layer (Fig. 2B). It remains in the NB layer for about 1 hour (Fig. 2, C and D) before moving dorsally and dividing to generate the dMP2 and vMP2 neurons (Fig. 2E). Soon after this cell division, the dMP2 and vMP2 express *ftz* at different levels; dMP2 consistently expresses *ftz* at a higher level than does vMP2 (Fig. 3). Expression of *ftz* disappears in the dMP2 and vMP2 neurons between hours 8 and 9 of development, before axonogenesis.

MP1 enlarges from the midline ventral ectoderm about 2 hours later than MP2, and begins to express *ftz* immediately (Fig. 4B); both of its progeny express *ftz* equally (Figs. 2E and 3). The last time *ftz* expression can be detected in the MP1 neurons is around hour 8 of development.

Neuroblast lineages. In *Drosophila* neurogenesis the segregation of 16 NB's in each thoracic hemisegment (*2*) and a single unpaired median NB has been reported (*8, 22*). We can identify 25 NB's in each thoracic hemisegment (Fig. 5) and the unpaired median NB. None of these NB's express *ftz*. However, *ftz* is expressed in specific GMC's which are the progeny of some of these NB's (Figs. 3 and 5), as do the neurons that arise from these GMC's. Here we focus on the neuronal progeny from two GMC's: (i) the first GMC (GMC-1) from NB 1-1, which divides to give rise to the aCC and pCC neurons (*23*); and (ii) a GMC (most likely GMC-1) from an unknown NB, which divides to give rise to the RP1 and RP2 neurons (*24*). NB 1-1 does not express *ftz*, but its first daughter, GMC-1, expresses *ftz* immediately after it is born (Figs. 3 and 4A). At about hour 6 of development, GMC-1 divides to generate the aCC and pCC neurons. Both neurons initially express *ftz* equally (Fig. 2E), but by hour 8 of development the aCC neuron expresses *ftz* at a higher level than the pCC neuron (Figs. 3 and 4B). A similar asymmetry is seen in the RP1 and RP2 neurons: RP2 expresses *ftz* at a higher level than does RP1 (Fig. 3).

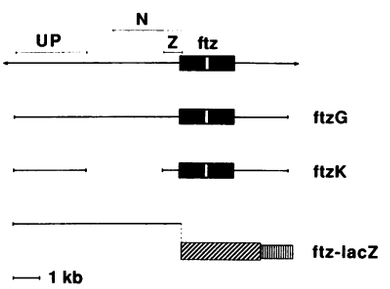


Fig. 1. Diagram of *ftz* P-element constructs and deletions. The top line shows the structure of the *fushi tarazu* (*ftz*) gene. Exons are shown by solid boxes, intron by an open box. Transcription is from left to right. Three control elements of the *ftz* gene are indicated: UP, the upstream element (zebra stripe enhancer); N, the neurogenic element; Z, the zebra element (*18*). Sequences from the *ftz* gene that are included in the P-element constructs *ftzG* and *ftzK* are shown in the middle. The *ftzG* construct contains all three control elements at the 5′ side of the transcription unit. The *ftzK* construct has the upstream and zebra elements, but has a deletion of a 2.8-kb region containing the neurogenic element (and an adjacent 0.95-kb fragment dispensable for normal *ftz* function) (*18*). In both constructs, *ftz* sequence extends approximately 2 kb from the polyadenylation signal. Structure of the *ftz/lacZ* fusion gene (corresponding to P[*ry*, *ftz/lacC*] element (*18*) is shown at the bottom. Solid line represents sequences from the *ftz* gene. *Escherichia coli lacZ* gene is represented as a cross-hatched box; the vertical hatched box indicates terminator sequences from the *Drosophila hsp70* gene. The indicated fragments were cloned into the Carnegie 20 P-element vector with the $rosy^+$ gene at the 5′ side of the *ftz* gene.

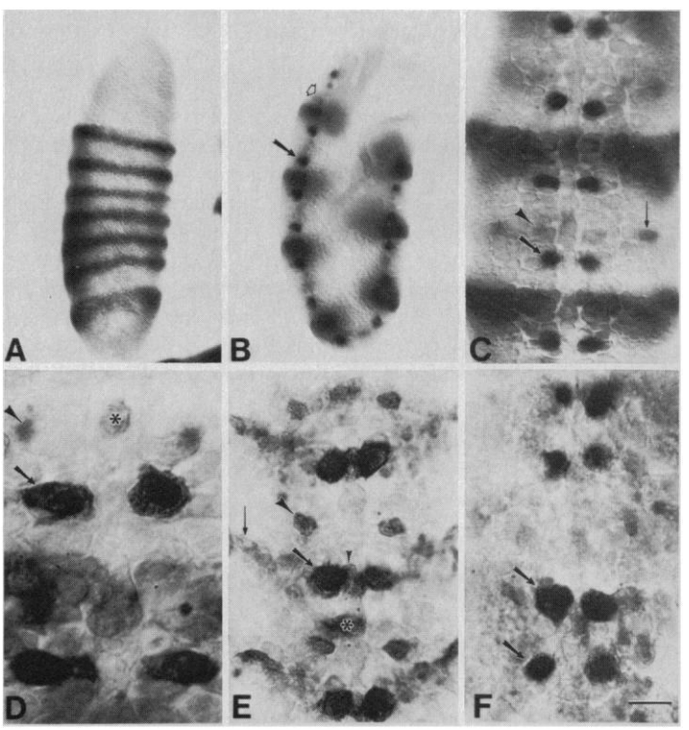


Fig. 2. Spatial pattern of *ftz* expression in transformant flies expressing *ftz/lacZ* gene. (A to E) Whole-mount wild-type (*ftz/lacZ*) and (F) *ftz* mutant ($ftz/lacZ;ftz^{w20}$) embryos stained with an antibody to β-galactosidase (β-gal). In all panels, anterior is up and the following symbols are used: MP2 and its progeny, wide black arrow; lack of MP2 in the second cephalic segment, hollow arrowhead; MP1 neurons, small arrowhead; the sibling aCC and pCC neurons, large arrowhead; glial precursor and its progeny, thin arrow. (**A**) Lateral view of a gastrulating embryo showing the early pattern of seven stripes. (**B**) Lateral view of a germ band extended embryo at about hour 5. Residual β-gal in the seven stripes is visible (by this time the *ftz* protein in the stripes has disappeared); the MP2 has just delaminated from the ventral ectoderm in segments C3 (third cephalic) to A8 (eighth abdominal). In C1, C2, and A9 there does not appear to be a MP2 precursor. (**C**) Ventral view of an embryo at about hour 6. The MP2's in segments C3-A1 are shown, as well as the glial precursor and the aCC and pCC. (**D**) High magnification ventral view of the MP2 cells in an hour 6 embryo. Also visible, but slightly out of the focal plane, are the aCC and pCC. The median neuroblast (*) stains transiently (the MNB cannot be detected with *ftz* antibody). (**E**) Ventral view of an embryo at about hour 7. MP2 has divided into its two progeny, dMP2 and vMP2 (wide black arrow). (**F**) Ventral view of the pattern of β-gal in an hour 6 $ftz/lacZ;ftz^{w20}$ embryo. Each double-wide segment contains two pairs of MP2's separated along the anterior-posterior axis by two neuroblasts (other embryos show 0 to 2 NB's between the MP2's). Scale bar: (A and B) 50 μm; (C) 20 μm; (D) 10 μm; (E and F) 15 μm.

In order to obtain an estimate (lower limit) for how many NB lineages produce *ftz*$^+$ progeny, we first examined embryos with the *ftz* antibody to identify the NB's with *ftz*$^+$ GMC's in contact with their dorsal surface (and thus likely to be derived from that NB); we counted eight NB's in each hemisegment (Fig. 5).

We also examined populations of single cells cultured in vitro from dissociated transformant embryos (4 to 6 hours) containing the *ftz/lacZ* fusion gene (*25*). In these cultures, individual NB's divide to form clonal clusters of neurons; thus the clusters of neurons represent the progeny of individual NB lineages (*26*). Approximately 35 percent of the neuronal clusters in vitro express β-gal, implying that about 8 to 9 of the 25 NB's per hemisegment produce progeny expressing *ftz*. These results also show that different lineages express *ftz* in different numbers of neurons. Of the clusters containing β-gal–positive neurons, some had as few as a single pair, whereas other clusters were composed entirely of β-gal–positive neurons.

Taken together, the results from both direct observations and cultured neuronal cells suggest that about 8 of the 25 NB's produce *ftz*$^+$ progeny, and that each of the *ftz*$^+$ NB lineages may have a characteristic pattern of *ftz* expression.

Glial lineages. Immediately after *ftz* expression is first detected in MP2, *ftz* is expressed in a large lateral cell (called the glial precursor or GP) (Figs. 2C and 5). Although this cell looks like an NB in both size and position within the NB array, the GP moves dorsally from the NB layer and divides symmetrically (unlike an NB) to form a pair of cells at about hour 6 of development (Fig. 2E). At between hour 7 and 7.5 of development additional cells are seen nearby; these might be progeny of a second GP, or the result of continued division of the initial progeny from the GP. These lateral cells assume a fibroblastic shape (Fig. 2E) characteristic of certain insect glia (*27*), and migrate anteriorly and medially along the dorsal surface of the developing CNS. In 12-hour *ftz/lacZ* embryos, β-gal is occasionally observed in identified glial cells lying along the dorsal surface of the CNS.

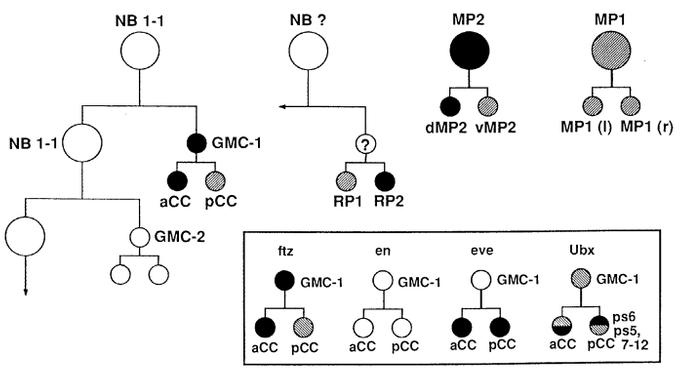


Fig. 3. Schematic diagram of four neuronal lineages that contain *ftz*$^+$ neurons. (Left) Neuroblast 1-1 (NB 1-1) is a stem cell that generates a family of ganglion mother cells (GMC's). GMC-1 expresses *ftz* immediately after its birth; it divides to form the aCC and pCC neurons, which express *ftz* at different levels (black, high level; cross-hatched, low level) soon after birth. (Left center) A GMC (most likely GMC-1) from an unknown NB divides to generate the RP1 and RP2 neurons, which express *ftz* at different levels. (right center) Midline precursor 2 (MP2) concurrently delaminates from the neuroectoderm and begins expressing *ftz*; its two neuronal progeny, the dorsal MP2 and ventral MP2 neurons (dMP2 and vMP2) express *ftz* at different levels. (Right) Midline precursor 1 (MP1) delaminates from the neuroectoderm and begins expressing *ftz*. Its two neuronal progeny, the MP1 neurons, are bilateral homologs; they express *ftz* at an equal level. (Inset) Level of expression of *ftz*, *en*, *eve*, and *Ubx* proteins in GMC-1 and its progeny aCC and pCC neurons. *ftz* and *eve* are expressed in aCC and pCC neurons in every segment (C1 to A8). *Ubx* is expressed in aCC and pCC neurons only in segments T2 to A6 (parasegments 5 to 12); it is expressed at a higher level in aCC than pCC in all of these segments, with the exception of segment T3 (parasegment 6) where expression is reversed (high in pCC and low in aCC). In segment T2 (parasegment 5) *Ubx* expression is usually equal in both aCC and pCC, although the aCC occasionally appears to express it at a higher level. In segment A6 (parasegment 12) *Ubx* expression in these two neurons is much lower than in the other abdominal segments.

Correlation of *ftz* CNS expression with cell determination. In the grasshopper, NB's (and probably MP's) are positionally determined as they enlarge; NB progeny are determined by lineage at the time of their birth (*5, 6*). The transient CNS expression of *ftz* in *Drosophila* correlates with both of these events. The *ftz* protein is apparently the first molecular marker that demonstrates the individual specification of neuronal precursor cells as they enlarge and delaminate from the ventral ectoderm (MP2 and MP1), or as they arise from specific NB's (such as GMC-1 from NB 1-1).

The quantitative *ftz* expression in particular neurons also correlates with patterns of determination. Sibling neurons that differentiate identically (such as the two MP1 progeny) express *ftz* protein at equal levels, whereas sibling neurons that follow different developmental pathways (such as aCC and pCC, vMP2 and dMP2, and RP1 and RP2), express different levels of *ftz* protein in each neuron (Fig. 3). These results are consistent with the idea that *ftz* plays a role in the control of neuronal determination.

Construction of transformant mutant embryos lacking *ftz* CNS expression. Having identified *ftz*$^+$ neurons in the developing CNS, we next examined the role that this gene plays in their development. In principle, the function of a gene can be analyzed by examining the phenotypes of mutations in the gene. However, to test the function of the *ftz* gene in the developing CNS, we could not simply examine embryos homozygous for a *ftz* null allele. The segmentation pattern defects in such embryos produce a distorted neuroectoderm showing displaced and duplicated neuronal precursors (*6, 13, 28*) (Fig. 2F). This pattern alteration makes it difficult to interpret defects that are due to loss of *ftz* expression in the CNS. Thus, to test *ftz* function during neurogenesis, we selectively removed the expression of *ftz* in the developing CNS by creating a promoter mutation of the *ftz* gene.

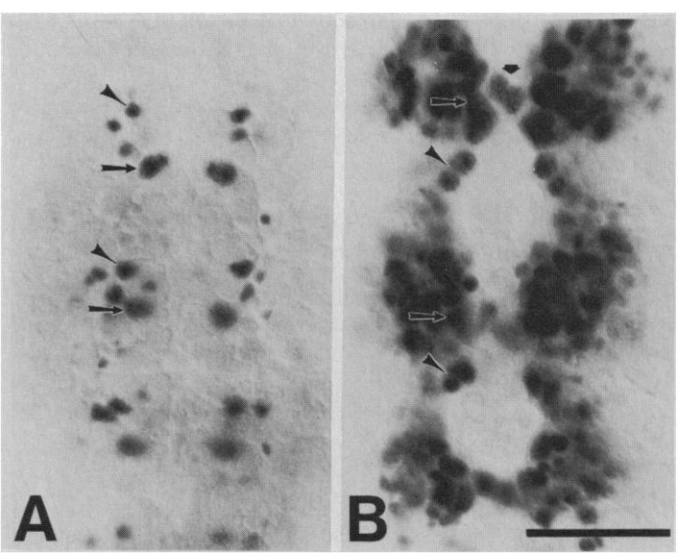


Fig. 4. Expression of *ftz* protein in the developing CNS. Ventral views of whole-mount embryos; anterior is up. (**A**) Germ band extended embryo about hour 5.5; there are approximately 6 GMC nuclei visible per hemisegment. The MP2 precursor (long arrow) and the first GMC from NB 1-1 (arrowhead) are visible. (**B**) Higher magnification of an embryo just prior to germ band shortening, about hour 8. The dMP2 and vMP2 neurons (long arrow), MP1 neurons (short arrow), and the aCC and pCC neurons (arrowhead) are indicated. Note the anterior migration of the aCC and pCC from near the posteriorly located MP2 neurons (A) to near the anteriorly located MP2 neurons (B). Scale bar is 25 μm.

We based our experiments on the previous identification of sequences required for *ftz* expression and function (*18*). The functional unit of the *ftz* gene lies within an approximately 10-kb sequence as assayed by rescue of lethality. The *ftz/lacZ* fusion genes have previously been used to identify three cis-acting control elements in the 5′ flanking region: the zebra element, which confers the striped blastoderm pattern; the neurogenic element, which is involved in CNS expression; and the upstream element, which has an enhancer-like effect on the expression of the striped pattern. The actions of the upstream element and the neurogenic element are independent; the neurogenic element is required only for the CNS expression but not for the blastoderm expression (*28*).

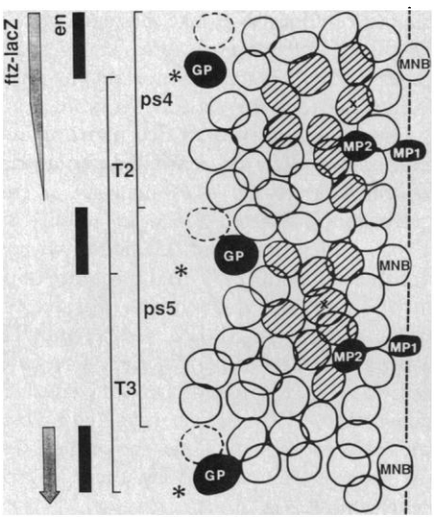


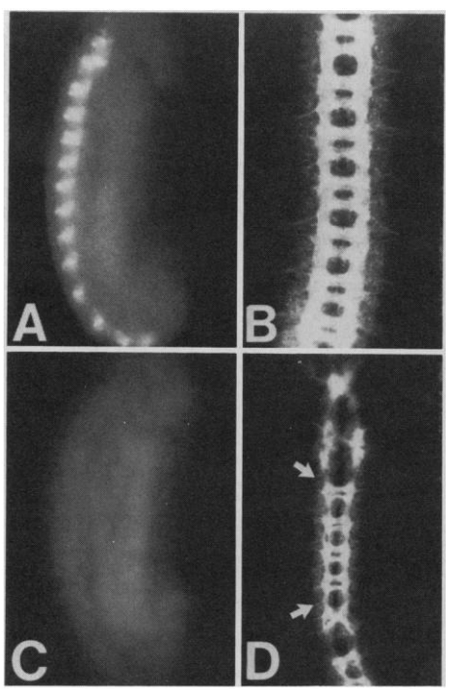
Fig. 5. Map of neuronal and glial precursor cells in the thoracic segments of the *Drosophila* embryo, based on camera lucida tracings. Segmental and parasegmental identities are to the left of the neuroblasts; the expression pattern of *ftz/lacZ* and *en* in the epidermis overlying the neuroblasts is shown at the far left. The precursor cells expressing *ftz/lacZ* are in black, the neuroblasts that produce ftz^+ progeny are cross-hatched. Neuroblast 1-1 is marked with an "X" and the possible 26th NB in each hemisegment is indicated by a dashed outline. The ventral midline (vertical dashed line) and tracheal pits (*) are indicated. MNB, median neuroblast; GP, glial precursor; MP2, midline precursor 2; MP1, midline precursor 1; T2 and T3 refer to the second and third thoracic segments, respectively; ps, parasegment.

We constructed a P element containing a mutated *ftz* gene (termed the *ftzK* gene) in which the neurogenic element is deleted from the 5′ flanking region (Fig. 1) and obtained six transformant lines carrying the *ftzK* gene by means of P element–mediated germline transformation (*29*). We compared our results from these lines with similar lines carrying the *ftzG* gene that contains all three cis-acting control elements (*18, 29*) (Fig. 1). Both *ftzK* and *ftzG* transformant lines were crossed into a ftz^{9H34} or ftz^{W20} (subsequently termed ftz^-) background lacking *ftz* protein, as assayed with a *ftz* antibody (*15*).

We monitored the expression of the *ftzG* and *ftzK* genes in a ftz^- background by an immunocytochemical assay of the *ftz* protein. Embryos carrying the *ftzG* gene show the normal pattern and levels of *ftz* protein distribution in both blastoderm stripes and in the developing CNS. Transformant mutant embryos containing the *ftzK* gene, in contrast, show either a decrease in or no detectable *ftz* protein in the CNS, depending upon the chromosomal position of the P element insertion. Similar line-dependent position effects influencing the quantitative expression have also been observed with *ftz/lacZ* genes (*18*).

For subsequent experiments we used the *ftzK.6* line which has nearly normal levels of *ftz* blastoderm expression without immunocytochemically detectable *ftz* protein in the CNS (Fig. 6). All *ftzK.6*; ftz^- individuals die before reaching the second instar larval stage, having either the normal cuticle pattern or weak segmentation defects (*30*). The defects ranged from a barely detectable loss of denticles in the fifth abdominal segment to the loss of all denticles in the first and fifth abdominal segments.

Expression of other segmentation and homeotic genes in the absence of *ftz* CNS expression. At blastoderm stage, *ftz* expression overlaps with that of several segmentation and homeotic genes,



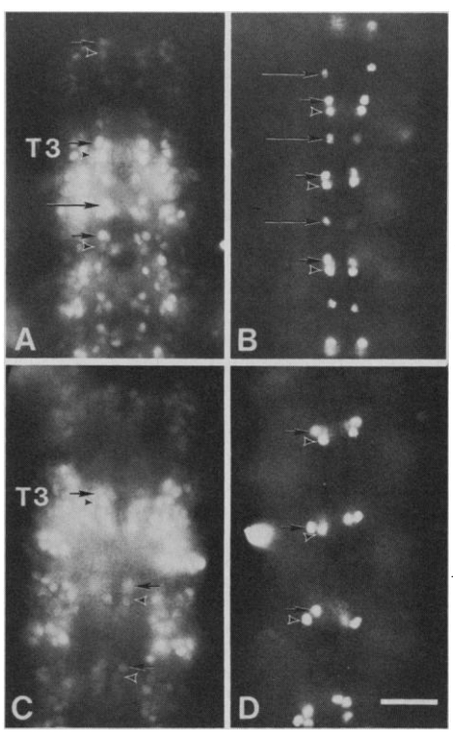


Fig. 6 (**left**). The *ftzK.6* embryos lack *ftz* CNS expression. (**A** and **B**) Wild-type embryos. (**C** and **D**) *ftzK.6*;ftz^{9H34} embryos. (A and C) Expression of the *ftz* protein in whole-mount embryos at 10 hours; lateral view, anterior is up. (B and D) Axons of the CNS in dissected embryos stained with an antibody to horseradish peroxidase (*43*) that stains all *Drosophila* axons. The *ftzK.6*;ftz^{9H34} embryo in (D) is an example with a major segmentation defect; most of the embryos we assayed had defects that were much less severe. Fusions of the T1/T2 segments are shown above the arrow and the A4/A5 segments are below bottom arrow. Segments from T3 to A3 (between arrows) show relatively normal morphology. Normal segments such as these were used to assay the phenotypes of individual identified neurons. Scale bar: (A and C) 100 μm; (B) 50 μm; (D) 90 μm. **Fig. 7** (**right**). *Ubx* and *eve* expression in identified neurons in the presence or absence of *ftz* CNS expression. Wild-type (**A** and **B**) and *ftzK.6*;ftz^- (**C** and **D**) embryos dissected to reveal the CNS stained with antibodies to *Ubx* (A and C) and *eve* (B and D) proteins. Anterior is up; the aCC (short arrow), pCC (arrowhead), and RP2 (long arrow) are indicated. (**A**) In segment T3 (parasegment 6) the aCC has a higher level of *Ubx* expression than the pCC; in more posterior segments (A1–A6) the relative levels of *Ubx* in each neuron is reversed, due to the large decrease in *Ubx* expression in the pCC. In segment T2 (parasegment 5) both aCC and pCC show an equal level of *Ubx* expression. (**B**) aCC, pCC, and RP2 neurons in all segments (C1–A8) show an equal level of *eve* expression. (**C**) In flies lacking *ftz* CNS expression, *Ubx* expression is normal in the aCC and pCC neurons, but absent in the RP1 and RP2 neurons (compare A to C). (**D**) In the absence of *ftz* CNS expression, *eve* expression is normal in the aCC and pCC neurons, but absent in the RP2 neuron (compare B to D). Scale bar is 50 μm.

including *engrailed* (*en*), *Ultrabithorax* (*Ubx*), and *even-skipped* (*eve*). In the absence of blastoderm *ftz* expression, the pattern of *en* and *Ubx* is altered (*17, 31*), while the pattern of seven stripes of *eve* expression is not affected (*32*). Since all three of these genes are expressed in a subset of cells during neurogenesis (*33–35*), we examined the expression of these genes in individually identified neurons (aCC, pCC, MP1, dMP2, vMP2, RP1, and RP2) (*20, 22*) in both wild-type embryos and those lacking *ftz* CNS expression.

Ubx is expressed in many embryonic neurons in a segment-specific pattern. In segments of the posterior thorax and abdomen [or more specifically, parasegments 5–12; see (*36*)], *Ubx* protein is expressed in the nuclei of the aCC, pCC, RP1, and RP2 neurons, but not in the MP1, dMP2, or vMP2 nuclei (Figs. 3 and 7). In the third thoracic (T3) segment (parasegment 6), *Ubx* is expressed at a higher level in the pCC neuron than the aCC neuron, whereas in the T2 and A1–6 segments (parasegments 5 and 7–12), its relative level of expression in these two neurons is the opposite (Fig. 3). The *en* protein is expressed in about 18 CNS nuclei per hemisegment in a segmentally repeated pattern (*37*); none of these neurons express *ftz*. The *eve* protein is expressed equally in the aCC, pCC, and RP2 neurons, but not in the MP1, dMP2, vMP2, or RP1 neurons (Figs. 7 and 8). Thus, three identified neurons (aCC, pCC, and RP2) each express *ftz*, *Ubx*, and *eve* proteins, and one (RP1) coexpresses *ftz* and *Ubx*.

In the absence of *ftz* CNS expression (in *ftzK.6*;*ftz*$^-$ embryos), the patterns of *en* expression are unaltered. In contrast, loss of *ftz* expression alters the neuronal expression pattern of both *Ubx* and *eve* proteins. The *Ubx* and *eve* genes respond to loss of *ftz* expression in different ways in different neurons. In *ftzK.6*;*ftz*$^-$ embryos, the aCC and pCC neurons express *Ubx* and *eve* at normal levels. The RP1 and RP2 neurons, however, no longer express the *Ubx* protein (Figs. 7C and 8). Moreover, the RP2 neuron does not express the *eve* protein (Figs. 7D and 8). These results indicate that, in one pair of neurons, *ftz* is required for the expression of *Ubx* and *eve* in RP2 and *Ubx* in RP1, whereas in another pair of neurons (aCC and pCC), it is not required for the expression of either gene. Thus, during segmentation, *ftz* regulates *en* and *Ubx* expression, but not *eve* expression; during neurogenesis, however, these regulatory interactions are different as *ftz* is required for the normal expression of *eve* and *Ubx*, but not *en*.

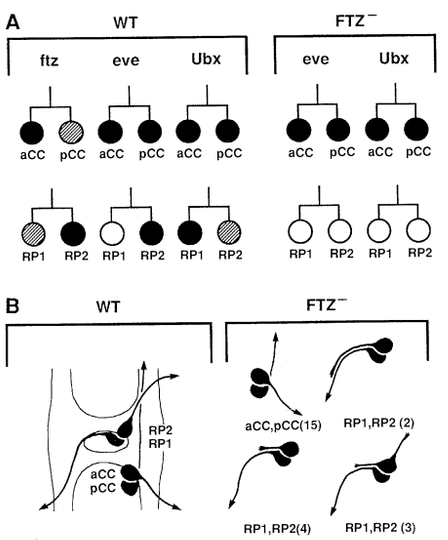


Fig. 8. Function of *ftz* CNS expression. (**A**) Expression of *ftz*, *eve*, and *Ubx* in four identified neurons (two pairs of siblings: aCC and pCC, RP1 and RP2), and their differential responses to the absence of *ftz* expression. High expression is indicated by black, lower expression by cross-hatching, and no expression by open circle (relative levels of *Ubx* expression vary from segment to segment; see Fig. 3). In the absence of *ftz* CNS expression, *eve* and *Ubx* are expressed normally in the aCC and pCC, but not in the RP1 and RP2 neurons. (**B**) Morphology of these same four neurons in wild-type embryos (left) and after removal of *ftz* CNS expression (right). Camera lucida tracings of intracellular dye fills with Lucifer yellow illustrate the normal pathway choice by the growth cones of the aCC, pCC, and RP1 neurons in the absence of *ftz*. However, the growth cone of the RP2 neuron does not behave normally. Three different morphologies have been observed: (i) duplication of RP1, (ii) short axon following the RP1 pathway, and (iii) two short axons, one following the RP1 pathway and the other following the normal RP2 pathway. The number of times the result was observed is shown in parentheses; normal RP2 morphology was never observed in the absence of *ftz* CNS expression.

Other evidence supports the notion that the regulatory mechanism of *ftz* gene expression differs in the CNS and the blastoderm. Use of the *ftz/lacZ* fusion genes reveals that high levels of *ftz* expression in the blastoderm stripes require interaction of the *ftz*$^+$ product with the upstream enhancer element, whereas such an interaction does not take place in *ftz*$^+$ cells in the CNS (*28*). Therefore, cell-specific expression of segmentation and homeotic genes in the CNS may be mediated by regulatory elements different from those used for blastoderm expression.

Development of identified neurons in the absence of *ftz* CNS expression. We next examined the axonal morphology of seven identified (normally *ftz*$^+$) neurons in embryos lacking *ftz* CNS expression. Dissected *ftzK.6*;*ftz*$^-$ embryos were viewed with Nomarski optics, and identified neurons were microinjected with the dye Lucifer yellow (*38*). In the absence of *ftz*, normally *ftz*$^+$ neurons survive (*28*) and differentiate. For six of the neurons examined (aCC, pCC, MP1, dMP2, vMP2, and RP1), axonal morphology was unaltered by loss of *ftz* expression (*n* is more than 15 for each neuron) (Fig. 8). In addition, we were able to identify morphologically normal aCC neurons in *ftz*$^-$ embryos lacking both blastoderm and CNS expression. Thus, the primary axonal outgrowth of these six neurons is unaltered by their loss of *ftz* expression during neurogenesis.

In contrast, the axon of the RP2 neuron does not behave normally ($n = 9$) (Fig. 8). In wild-type embryos, the RP2 axon extends anterior and then laterally out along the ipsilateral intersegmental nerve at the anterior border of its own segment, whereas its sibling's axon (RP1) extends across the midline, and then posterior and laterally out along the contralateral intersegmental nerve at the posterior border of its own segment (*22, 39*). Intracellular dye injections reveal that, in *ftzK.6*;*ftz*$^-$ embryos, the RP2 growth cone either follows the same pathway as its sibling's axon (RP1) or, alternatively, has two growth cones, one following the RP1 pathway and the other extending anteriorly (Fig. 8). Thus, in the absence of *ftz* (and *eve*), RP2 appears to be partially transformed toward its sibling, RP1.

Segmentation genes, the homeobox, and neurogenesis. Neuronal differentiation is a complex process involving many different events, including the choice of appropriate axon pathways, dendritic branching, synaptic partners, neurotransmitter (or transmitters), receptors, and ion channels. We have not been able to assay for properties other than axonal morphology. However, the pathway choice by the neuron's primary growth cone is a complex and stereotyped event that occurs early in neuronal differentiation and thus serves as a good indication of cellular determination.

In the absence of *ftz*, the axon morphology of the RP2 neuron is transformed toward that of its sibling, the RP1 neuron. This transformation suggests that loss of *ftz* expression has caused an alteration in the identity of the RP2 neuron. Such a transformation in cell fate is similar to the consequences of homeotic and segmentation mutations observed in the epidermis (*9, 10*) and supports the idea that *ftz* plays a regulatory role in neuronal determination as well as in cell determination during segmentation.

Of the seven identified neurons described here, which normally express *ftz*, we have observed a mutant phenotype in only one of them (RP2). This may mean that there is some redundancy in the system such that the absence of *ftz* is compensated for by the presence of another gene (or genes) product which also serves to

control the identity of these neurons. The fact that the RP1 neuron, which fails to express *Ubx* protein in the absence of *ftz* CNS expression, is morphologically normal indicates that the expression of the *Ubx* gene in this neuron is also not required for normal axonal morphology. Whether or not lack of *ftz* (or *Ubx*) expression affects other cellular properties in those neurons with normal axonal morphology awaits future analysis.

The very cell that shows a mutant phenotype in the absence of *ftz* expression is the same one that shows an altered expression of another segmentation gene, *eve*. This may suggest that these two genes perform an overlapping function in determining neuronal identity, and that the mutant phenotype is observed only when the expression of both genes is absent. Alternatively, the effect of *ftz* on the identity of this neuron may be mediated via the *eve* gene.

The *ftz* gene is expressed in a specific subset of about 30 neurons in each hemisegment, and *eve* in a different but overlapping subset of neurons. In this regard, *ftz* and *eve* are not alone; many other segmentation genes are expressed in the developing nervous system in a segmentally repeated pattern that differs from their earlier striped pattern (*34, 35, 37, 40*). These genes may be involved in both regulating the neuronal expression of each other, as well as in controlling neuronal fate by regulating the expression of downstream genes such as those encoding surface recognition molecules. Whether the set of known segmentation genes is sufficient to control the identity of about 250 different neurons in each hemisegment, or whether additional genes are required, remains to be determined.

The two segmentation genes discussed here, *ftz* and *eve*, both contain the homeobox (*11, 12, 32*). Not only are they and other *Drosophila* homeobox genes expressed during neurogenesis, but in addition, several homeobox genes thus far cloned in vertebrates appear to be primarily expressed in the nervous system (*41*). Furthermore, *mec-3*, a gene required for the differentiation of the touch receptor neurons in the nematode *Caenorhabditis elegans*, contains a homeobox (*42*). These observations may provide clues to the evolution of these genes. It is quite likely that this protein motif evolved before the split of the protostomes and deuterostomes, and possibly before the independent evolution of segmentation in both phyletic lines. Furthermore, it is likely that the molecular mechanisms for creating neuronal diversity and specificity arose very early in metazoan evolution. Thus, one intriguing possibility is that the homeobox-containing genes may have had an early evolutionary role in the control of neuronal fate, with this role subsequently maintained in both independent evolutionary lines.

REFERENCES AND NOTES

1. C. M. Bate, *J. Embryol. Exp. Morphol.* **35**, 107 (1976); C. Q. Doe and C. S. Goodman, *Dev. Biol.* **111**, 193 (1985).
2. V. Hartenstein and J. Campos-Ortega, *Wilhelm Roux's Arch. Dev. Biol.* **193**, 308 (1984).
3. C. M. Bate and E. B. Grunewald, *J. Embryol. Exp. Morphol.* **61**, 317 (1981).
4. C. S. Goodman and N. C. Spitzer, *Nature (London)* **280**, 208 (1979); J. A. Raper *et al.*, *J. Neurosci.* **3**, 20 (1983); P. H. Taghert, C. S. Goodman, *ibid.* **4**, 989 (1984).
5. C. Q. Doe and C. S. Goodman, *Dev. Biol.* **111**, 207 (1985); J. Y. Kuwada and C. S. Goodman, *ibid.* **110**, 114 (1985); C. Q. Doe, J. Y. Kuwada, C. S. Goodman, *Philos. Trans. R. Soc. London B* **312**, 67 (1985).
6. C. Q. Doe, C. S. Goodman, *Cold Spring Harbor Symp. Quant. Biol.* **50**, 891 (1985).
7. R. Lehmann *et al.*, *Wilhelm Roux's Arch. Dev. Biol.* **192**, 62 (1983).
8. J. A. Campos-Ortega, *Trends Neurosci.* **8**, 245 (1985).
9. C. Nüsslein-Volhard and E. Wieschaus, *Nature (London)* **287**, 795 (1980); M. P. Scott and P. H. O'Farrell, *Annu. Rev. Cell Biol.* **2**, 49 (1986); J. P. Gergen, D. Coulter, E. Wieschaus, *Symp. Soc. Dev. Biol.* **44**, 195 (1986).
10. E. B. Lewis, *Nature (London)* **276**, 565 (1978); T. C. Kaufman and M. Abott, in *Molecular Aspects of Early Development*, G. Malacinski and W. Klein, Eds. (Plenum, New York, 1984), p. 189.
11. A. J. Weiner and M. P. Scott, *Cell* **37**, 843 (1984); A. Kuroiwa, E. Hafen, W. J. Gehring, *Cell* **37**, 825 (1984).
12. A. Laughon and M. P. Scott, *Nature (London)* **310**, 25 (1984).
13. B. Wakimoto, F. R. Turner, T. C. Kaufman, *Dev. Biol.* **102**, 147 (1984).
14. J. C. W. Shephard *et al.*, *Nature (London)* **310**, 70 (1984); C. Desplan, J. Theis, P. H. O'Farrell, *ibid.* **318**, 630 (1985).
15. S. B. Carroll and M. P. Scott, *Cell* **43**, 47 (1985).
16. E. Hafen, A. Kuroiwa, W. J. Gehring, *ibid.* **37**, 833 (1984).
17. P. W. Ingham and A. Martinez-Arias, *Nature (London)* **324**, 592 (1986).
18. Y. Hiromi, A. Kuroiwa, W. J. Gehring, *Cell* **43**, 603 (1985).
19. H. Krause and W. Gehring, unpublished results.
20. Whole-mount embryo staining was done by a modification of the procedure of T. J. Mitchison and J. W. Sedat [*Dev. Biol.* **99**, 261 (1981)] to remove their vitelline membranes (all steps done at room temperature except as noted). Embryos were incubated in rabbit antiserum to β-gal (Cappel) at a dilution of 1:1000 in PBT (phosphate-buffered saline + 0.2 percent bovine serum albumin + 0.1 percent Triton X-100) overnight at 4°C, washed in PBT, and incubated for 30 minutes in PBT plus 5 percent normal goat serum. A 1:200 dilution of affinity-purified, horseradish peroxidase (HRP)–conjugated goat antiserum to rabbit immunoglobulin G (IgG) (Cappel) was added for a 2-hour incubation. The embryos were washed in PBT, and stained for peroxidase with diaminobenzidine (Sigma) at 0.5 mg/ml and 0.003 percent hydrogen peroxide. Embryos were transferred to ethanol, cleared in methyl salicylate, and viewed with Nomarski optics. Whole-mount embryos stained for *ftz*, *eve*, *Ubx*, and *en* protein were processed as above, with the following exceptions. Fixation was for 20 minutes in PEM-FA (0.1*M* Pipes, 2 m*M* EGTA, 1 m*M* $MgSO_4$, 4 percent formaldehyde, *p*H 6.95) (*15*); primary antibodies were rabbit antisera to *ftz* (*15, 19*) or *eve* (*35*), and mouse monoclonal antibodies to *Ubx* (*33*) or *en* (*37*). Secondary antibodies were goat antibody to rabbit IgG or goat antibody to mouse IgG conjugated with either fluorescein isothiocyanate (FITC) or HRP (Cappel). FITC-labeled embryos were washed, transferred to PBT with 0.15 percent ascorbic acid (to retard bleaching) at *p*H 8.8, and viewed with epifluorescence; HRP-labeled embryos were processed as described above.
21. The *ftz/lacZ* transformant line (43.04) carries four copies of the P[*ry*, *ftz/lacC*] gene (*18*). This line contains homozygous second and third chromosom that carry the P[*ry*, *ftz/lacC*].4 and the P[*ry*, *ftz/lacC*].1 insertion, respectively.
22. J. B. Thomas *et al.*, *Nature (London)* **310**, 203 (1984).
23. C. S. Goodman *et al.*, *Developmental Order and Regulation*, S. Subtelny and P. B. Green, Eds. (Liss, New York, 1982), p. 275; M. J. Bastiani *et al.*, *J. Neurosci.* **6**, 3518 (1986).
24. C. S. Goodman *et al.*, *Science* **225**, 1271 (1984).
25. Embryonic neuronal cultures were prepared by a modification of the method of Patel *et al.* (*39*) (also N. H. Patel and C. S. Goodman, unpublished results). Cultured neurons were fixed and immersed in assay buffer [J. A. Simon *et al.*, *Cell* **40**, 805 (1985)] containing 0.2 percent X gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma) and incubated for 4 to 12 hours.
26. A. Furst and A. P. Mahowald, *Dev. Biol.* **112**, 467 (1985).
27. M. J. Bastiani and C. S. Goodman, *J. Neurosci.* **6**, 3542 (1986).
28. Y. Hiromi and W. J. Gehring, *Cell* **50**, 963 (1987).
29. The *ftzK* construct was made by exchanging the 5′ flanking region of the *ftzG* construct with that of the *ftz/lacD* gene (*28*). The proximal boundary of the exchanged region is the Pst I site, located within the zebra element. Germline transformation was done as described in (*18*) and by G. M. Rubin and A. C. Spradling, [*Science* **218**, 348 (1982)]. The two *ftzG* transformant lines tested, P[*ry*, *ftzG*].5 and P[*ry*, *ftzG*].8, behaved similarly when tested for *ftz* protein expression and viability.
30. *ftzK.6*;*ftz*^{w20}/TM3 males were crossed to *ftzK.6*;*ftz*^{9H34}/TM3 virgin females to generate *ftzK.6*;*ftz*$^{-}$ embryos (*ftzK.6*;*ftz*^{w20}/*ftz*^{9H34}). The lethal phase of the *ftzK.6*;*ftz*$^{-}$ individuals was determined by collecting all embryos from a 6-hour collection, counting the number of hatched larvae (thus identifying the number of *ftzK.6*;*ftz*$^{-}$ larvae), culturing the larvae on yeast paste, and counting the survivors each day. Sixty percent of *ftzK.6*;*ftz*$^{-}$ individuals died as fully developed embryos, and 40 percent died as first instar larvae. Similar experiments with *ftzG* transformants show that 66 percent of the *ftzG*;*ftz*$^{-}$ individuals survived to adulthood.
31. K. Howard and P. Ingham, *Cell* **44**, 949 (1986).
32. K. Harding *et al.*, *Science* **233**, 953 (1986); P. M. Macdonald *et al.*, *Cell* **47**, 721 (1986).
33. R. A. H. White and M. Wilcox, *Cell* **39**, 163 (1984). P. A. Beachy, S. Helfand, D. S. Hogness, *Nature (London)* **313**, 545 (1985).
34. S. Dinardo, J. M. Kuner, J. Theis, P. H. O'Farrell, *Cell* **43**, 59 (1985).
35. M. Frasch, T. Hoey, C. Rushlow, H. Doyle, M. Levine, *EMBO J.* **6**, 749 (1987).
36. A. Martinez-Arias and P. A. Lawrence, *Nature (London)* **313**, 639 (1985).
37. C. Q. Doe, T. Kornberg, C. S. Goodman, unpublished results.
38. Embryos of *ftzK.6*;*ftz*$^{-}$ genotype could be identified as embryos having weak segmentation defects in the CNS. Neurons in morphologically normal segments were used for intracellular dye injections (*22*).
39. N. H. Patel, P. M. Snow, C. S. Goodman, *Cell* **48**, 975 (1987).
40. D. C. Knipple *et al.*, *Nature (London)* **317**, 40 (1985); D. Bopp *et al.*, *Cell* **47**, 1033 (1986); N. E. Baker, *EMBO J.* **6**, 1765 (1987); U. Gaul, E. Seifert, R. Schuh, H. Jäckle, *Cell* **50**, 639 (1987).
41. M. F. Awgulewitsch *et al.*, *Nature (London)* **320**, 328 (1986); A. Simeone *et al.*, *ibid.*, p. 763; A. E. Carrasco and G. M. Malacinski, *Dev. Biol.* **121**, 69 (1987); A. L. Joyner, G. R. Martin, *Genes Dev.* **1**, 29 (1987); W. F. Odenwald *et al.*, *ibid.*, p. 482.
42. M. Chalfie and J. Sulston, *Dev. Biol.* **82**, 358 (1981); J. Way and M. Chalfie, personal communication.
43. L. Y. Jan and Y. N. Jan, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2700 (1982).

44. We thank N. Patel for doing the small-scale neuronal culture experiments; S. Carroll, M. Scott, and H. Krause for antibodies to *ftz*; M. Frasch and M. Levine for antibodies to *eve*, R. White for antibodies to *Ubx*, N. Gaye and T. Kornberg for collaboration in helping us make antibodies to *en*, and N. Patel, D. Smouse, and M. Scott for reading the manuscript. Supported by a NICHHD Developmental Biology Traineeship (C.Q.D.), American Cancer Society Postdoctoral Fellowship (Y.H.), Swiss National Science Foundation and the Kanton Basel Stadt (W.J.G.), and NIMH and NIH grants (C.S.G.).

10 September 1987; accepted 19 November 1987