

The NK-2 Homeobox Gene and the Early Development of the Central Nervous System of *Drosophila*

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My colleagues and I deciphered the genetic code gradually, over a period of five years, between 1961 and 1965. I then stopped working on the code and began working in the field of neurobiology. The logic that connects the genetic code to neurobiology is that information is processed in both genetic and neural systems.

My interest in the NK-2 homeobox gene¹ of *Drosophila* stems from the observation that NK-2 is the earliest predominantly neural gene regulator that has been found thus far that is expressed in the ventrolateral neurogenic an-

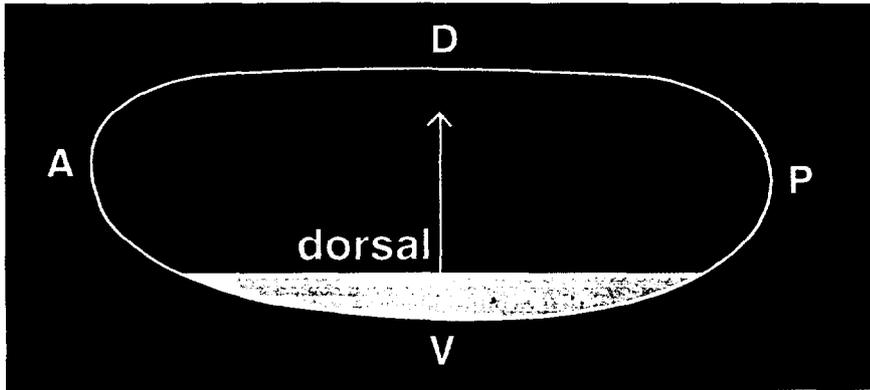


FIGURE 1. Side view of stage 5 *Drosophila* embryo illustrates concentration gradients of proteins that regulate gene expression. The concentration of bicoid homeobox protein is high in the anterior (A) region and low in the posterior (P) region of the embryo. A concentration gradient of nanos protein is established in the posterior to anterior direction. A concentration gradient of dorsal protein is established in nuclei in the ventral (V) towards the dorsal (D) region of the embryo.

lage, which gives rise to part of the central nervous system of the embryo. I will tell you what we know about the NK-2 gene and relate these findings to the early development of the *Drosophila* embryo and the central problem of understanding the principles that are used initially to construct part of the central nervous system of the embryo. The studies on NK-2 were performed by my colleagues, Yongsok Kim, Kohzo Nakayama, Noriko Nakayama, Dervla Mellerick, Lan-Hsiang Wang, Keith Webber, and Rajnikant Lad.

The nucleus of a fertilized *Drosophila* embryo undergoes 13 rounds of nuclear division in the first 130 minutes of embryonic development, resulting in an embryo that consists of a single cell with approximately 5,000 nuclei. Most of the nuclei move to the periphery during stage 4 (80–130 minutes after fertilization, nuclear divisions 10–13^{2,3}) and cell membranes form around each nucleus between 130 and 170 minutes after fertilization (stage 5). The anterior-posterior and ventral-dorsal axes of the embryo are established and different cell types are generated during stages 4 and 5 by the formation of concentration gradients of proteins that regulate gene expression (FIG. 1). A concentration gradient of bicoid homeobox protein is established in the anterior-posterior direction (high concentration in the anterior portion of the embryo, low concentration in the posterior portion⁴⁻⁶); and a concentration gradient of nanos protein is established in the posterior to anterior direction.⁷⁻¹⁰ Concomitantly, a concentration gradient of dorsal protein is established in nuclei, with the highest concentration of dorsal protein in nuclei in the ventral portion of the embryo and the lowest concentration in nuclei in the dorsal part of the embryo.¹¹⁻¹⁴ These gene regulators and terminal gene

regulators initiate the induction or repression of other genes that encode proteins that regulate gene expression and result in dynamically changing patterns of gene expression in different parts of the embryo, depending upon the concentrations of gene regulators that the nuclei were exposed to. The anterior-posterior gradients of gene regulators result in the formation of vertical stripes of equivalent nuclei that were exposed to the same concentrations of gene regulators, whereas the ventral to dorsal gradient of dorsal protein results in the formation of horizontal stripes of equivalent nuclei (for reviews see Refs. 15–18). In effect, the embryo is divided into a bilaterally symmetric checkerboard of clusters of nuclei that express different combinations of genes for proteins that regulate genes. The position of a nucleus in the embryo therefore determines the initial developmental fate of the nucleus.

In FIGURE 2 is shown the composite nucleotide sequence of NK-2 cDNA and genomic DNA and the deduced amino acid sequence of NK-2 protein.¹⁹ The NK-2 gene contains 3 exons and 2 introns; introns 1 and 2 are approximately 1.6⁸¹ and 3.1¹⁹ kb in length, respectively. 3 NK-2 protein contains two regions near the N-terminus that consist almost entirely of alternating acidic and basic amino acid residues or pairs of acidic and basic amino acid residues. The protein contains multiple Ala repeats, an Asn repeat, an acidic domain, followed by a homeodomain, which is not closely related to any other *Drosophila* homeodomain. The C-terminal region of NK-2 protein contains a 17-amino acid residue sequence of unknown function termed the NK-2 box (amino acid residues 631–647) that has been highly conserved during evolution and a His-Ala repeat. The NK-2 gene was shown to reside on the sex chromosome at ICI-5.¹

The NK-2 homeodomain has been conserved during evolution. FIGURE 3A compares the amino acid sequence of the *Drosophila* NK-2 homeodomain^{1,19} and NK-2-like homeodomains from *Xenopus*,²⁰ mouse,^{21,22} planaria,^{23,24} leech,²⁵ and tapeworm.²⁶ The similarity in amino acid sequence ranges from 95 to 67 percent. The mouse genome contains six copies of the NK-2 gene, presumably formed by gene duplication.

The amino acid residues that comprise NK-2 homeodomain α -helices I, II, and III were determined by NMR.²⁷ Binding of a 77-amino acid residue protein that contains the NK-2 homeodomain to a high-affinity NK-2 binding site in DNA results in an increase in the length of α -helix III from 11 to 19 amino acid residues (from NK-2 homeodomain residues 42–52 to 42–60) and also increases the stability of the secondary structure of the homeodomain.²⁷

Xenopus and mouse proteins with NK-2-like homeodomains also contain the highly conserved 17-amino acid residue NK-2 box after the homeodomain (94–77% homology with the NK-2 box sequence of *Drosophila* NK-2 protein) (FIG. 3B). The *Drosophila* NK-3^{1,29} (*bagpipe*)²⁸ homeodomain protein also contains a sequence related to the NK-2 box (47% homology); how-

A	SPECIES	α -HELIX 1			α -HELIX 2		α -HELIX 3			%	HOMOL- OGY	REF.	
		1	11	22	28	38	42	52	60				
	NK-2	d	KRKRRLVLF	TKAQT	YELERR	FRQRYLS	APEREHLAS	LIRLTP	QVKIWF	QNHRYKTK	RAQ	100	1, 19
	XeNK-2	x	-----S	-----	-----	-----	-----	-----	-----	-----M	-----R	95	20
	Nkx-2.2	m	-----S	-----	-----	-----	-----	-----	-----	-----	-----M	95	21
	Nkx-2.4	m	-----SQ	--V-----	-----K	--K-----	-----M	--H-----	-----	-----M	-----QA	83	21
	Nkx-2.1	m	R-----SQ	--V-----	-----K	--K-----	-----M	--H-----	-----	-----M	-----QA	82	21
	Nkx-2.3	m	R--P-----SQ	--VF-----	-----K	-----	-----SLK	--S-----	-----R	-----C	-----QR	75	21, 22
	Nkx-2.5	m	R--P-----SQ	--V-----	-----K	-----	-----DQ	--VLK	--S-----	-----R	-----C	73	22
	Nkx-2.6	m	Q--S-----SQ	--VLA-----	-----K	-----T	-----ALQ	--S-----	-----R	-----S	-----SQR	70	22
	Dth-1	p	-----S	-K-IL-----	H-----	KK-----	-----N	-G-S-----	-----	-----M	-----H	80	23, 24
	Dth-2	p	R----I--SQ	--I-----	-----K	--K-----	-----N	--N-----	-----	-----C	-----S	82	23, 24
	Lox 10	l	R----I--SQ	--I-----	-----K	-----	-----TF	--G-----	-----	-----	-----KSK	80	25
	EgHbx-3	t	QS-----N	-F-ISQ--K--	K--K--	T-Q-QE--	HT-G-----	-----	-----A	-----M	-----LF	67	26

B	SPECIES	ΔAA	% HOMOLOGY		REF.	
	NK-2	d	26	SPRRVAVPVLVR·NGKPC	100	1, 19
	XeNK-2	x	11	-----·D-----	94	20
	Nkx-2.2	m	11	-----·D-----	94	21
	Nkx-2.3	m	14	P-----·D-----	88	21
	Nkx-2.4	m	23	-----·K·D-----	88	21
	Nkx-2.1	m	34	-----·K·D-----	88	21
	Nkx-2.5	m	13	PA--I-----·D-----	77	22
	Nkx-2.6	m	13	PA-----L·D-----	77	22
	NK-3 (bagpipe)	d	10	ASK--P-Q----ED-STT	47	1, 28-29

FIGURE 3. (A) Comparison of the amino acid sequence of the (d) *Drosophila* NK-2 homeodomain with similar homeodomains from (x) *Xenopus*, (m) mouse, (p) planaria, (l) leech, and (t) tapeworm (from Ref. 19). The symbol (-) represents the same amino acid residue as NK-2. The amino acid residues of *Drosophila* NK-2 homeodomain α -helix 1, α -helix 2, and α -helix 3 were determined in NMR.²⁷ In the absence of DNA α -helix 3 extends from amino acid residue 42 through 52. However, binding of the NK-2 homeodomain to a high-affinity NK-2 site in DNA increases the length of α -helix 3 (residues 42-60).²⁷ (B) The NK-2 box is a highly conserved 17-amino acid residue sequence that is found after the homeodomain in proteins related to NK-2. Δ AA represents the number of amino acid residues between the end of the homeodomain and the beginning of the conserved NK-2 box sequence. The symbol (-) represents the same amino acid residue; (·) represents the absence of an amino acid residue.

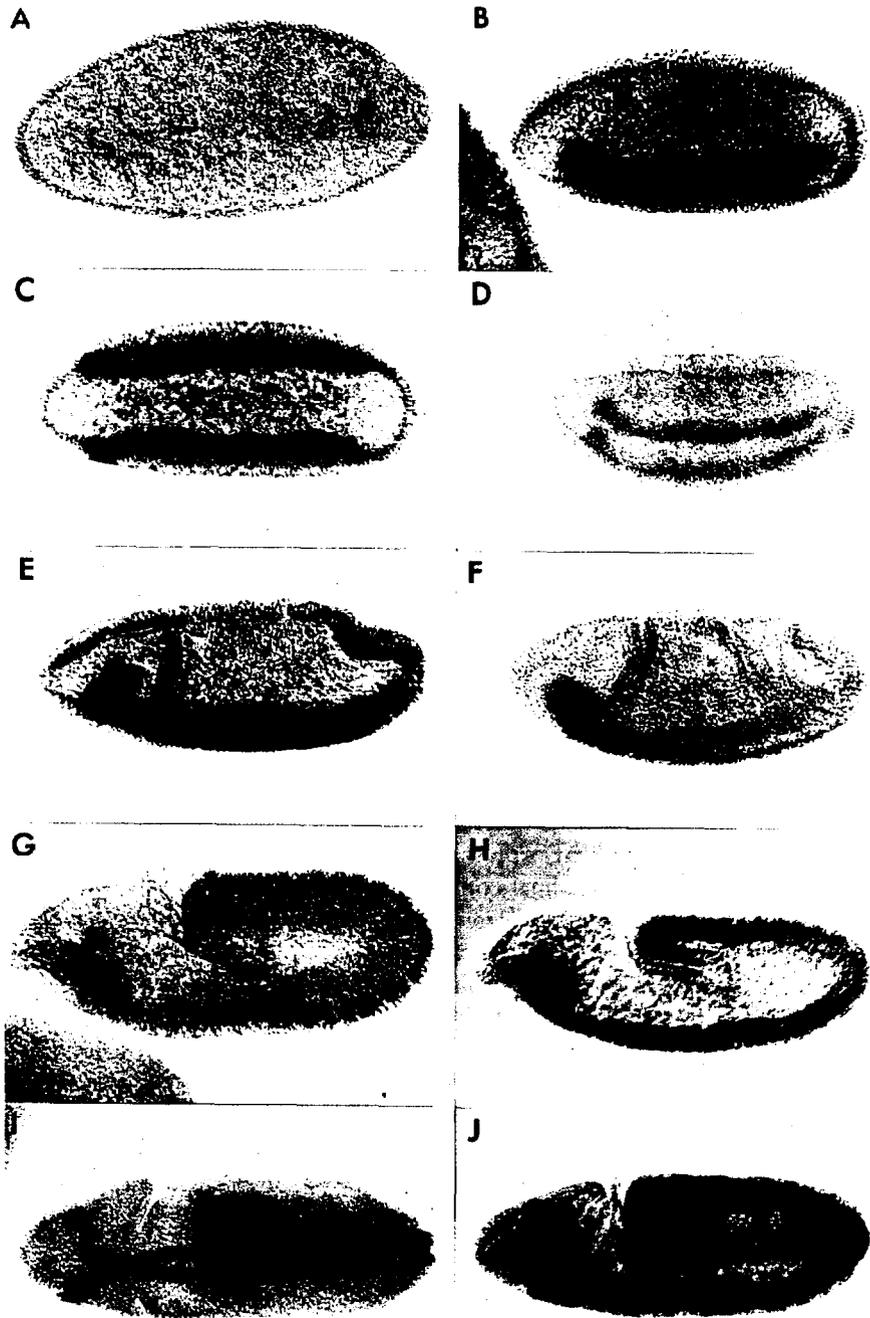
ever, a highly conserved NK-2 box was not detected in the planarian homeodomain proteins, Dth-1 or Dth-2.

Northern analysis of poly A⁺ RNA from *Drosophila* at various stages of development showed that NK-2 mRNA is present in highest concentration in 3-6-hr *Drosophila* embryos and then progressively decreases during further embryonic development.¹⁹ No NK-2 mRNA was detected in 0-3-hr *Drosophila* embryos; therefore, no maternal NK-2 mRNA was found. Larvae and pupae contain greatly reduced levels of NK-2 mRNA compared with that of 3-6-hr embryos; however, an increase in NK-2 mRNA was found in adult flies.

FIGURE 4 shows the distribution of NK-2 mRNA in *Drosophila* embryos as a function of developmental age, determined by *in situ* hybridization.¹⁹

NK-2 gene expression is initiated during stage 4 in bilaterally symmetrical longitudinal stripes, one stripe on each side, in the ventral (i.e., medial) half of the ventrolateral neurogenic anlage (FIG. 4A). By stage 5, when the first cell membranes are forming around the nuclei, the stripes of nuclei that express NK-2 extend from 0 to 90% of the embryo length and each stripe is 6 or 7 nuclei in width (FIG. 4B). NK-2 is also expressed in part of the procephalic neuroectodermal anlage, the endodermal anterior and posterior midgut anlagen, and the hindgut anlage. The ventral mesodermal primordium invaginates during gastrulation, bringing the longitudinal NK-2 positive bands of neuroectodermal cells closer to the ventral midline, separated only by ventral midline mesodermal cells, which do not contain NK-2 mRNA (FIGS. 4C–F). At first the level of NK-2 mRNA in the band of NK-2-positive cells is fairly homogeneous; however, during early gastrulation clusters of cells with high levels of NK-2 mRNA appear that are separated by vertical stripes of cells, 1 or 2 cells in width, that contain lower levels of NK-2 mRNA, apparently due to repression of the NK-2 gene (FIGS. 4E–F). Initially one cluster of cells with a high level of NK-2 mRNA is formed per hemisegment; later two clusters of NK-2-positive cells appear per hemisegment (FIGS. 4G–J). Germ band extension results in an increase in the length of the band of cells that synthesize NK-2 mRNA and a concomitant decrease in the width of the band to 2 to 3 cells per side (FIG. 4I). Hence, the neuroectodermal cells that synthesize NK-2 mRNA give rise to medial and paramedial neuroblasts that continue to synthesize NK-2 mRNA. Ganglion mother cells and neurons were found that express the NK-2 gene that perhaps are the progeny of neuroblasts that express the NK-2 gene. However, during later embryonic development, the abundance of NK-2 mRNA decreases in some neurons and is extinguished in others. Some neurons that express the NK-2 gene form commissures and others contribute to longitudinal connectives.

FIGURE 5 shows schematic diagrams of cross-sections of embryos at the cellular blastoderm stage (stage 5) before nuclei have been enclosed by cell membranes, and at the end of gastrulation (end of stage 7) after ventral mesodermal primordium cells have invaginated. These cross-sections of embryos illustrate ventral-dorsal patterning during early development of the *Drosophila* embryo. The ventral to dorsal concentration gradient of dorsal protein^{11–14} activates the *twist*^{30–34} and *snail*^{34–36} genes in the most ventral nuclei, which correspond to the mesodermal anlage, and the NK-2 gene is activated in the ventral (medial) half of the ventrolateral neuroectodermal anlage. A gene regulator specific for dorsal (lateral) neuroectoderm has not been identified thus far. Dorsal protein represses the *decapentaplegic (dpp)* gene,^{37–40} which encodes a protein that is a homologue of TGF- β ,^{37,41} and the *zen-1* and *zen-2* homeobox genes^{42–46} in nuclei in the ventral and lateral parts of the embryo, but not in nuclei that become dorsolateral epidermoblasts or dorsal amnioserosa,



respectively. Hence, the concentration gradient of dorsal protein establishes the ventral-dorsal axis of the embryo and divides the embryo into longitudinal bands of nuclei that have different developmental fates. After cell membranes form, the most ventral cells, the mesodermal anlage, invaginate, which brings the mesectodermal cells to the ventral midline.

Neuroectodermal cells gradually segregate as neuroblasts between about 3.5 and 7.3 hours after fertilization⁴⁷⁻⁴⁹ (FIG. 6). Eventually a monolayer of neuroblasts and glioblasts separated by ventral midline mesectodermal cells is formed above the epidermal cells. Doe⁴⁹ has shown that thirty-one neuroblasts or glioblasts delaminate per hemisegment, that each is a unique cell type, and that the relative position of each neuroblast or glioblast in the set is determined.

A *Drosophila* embryo contains 14 parasegments and additional segments in the head region. Approximately 800 ventrolateral neuroblasts or glioblasts segregate from the medial and lateral neuroectoderm per embryo and additional neuroblasts and glioblasts are formed from mesectodermal cells.⁴⁹ The pattern of neuroblasts is repeated in different segments possibly with some variation. However, some genes that encode proteins that regulate gene expression are known to be expressed only by cells in a single segment, or a few segments. Although most of the proof is lacking, it is likely that many neuroblasts also express segment-specific gene regulators and that most of the neuroblasts per side eventually will be found to be unique cell types.

Three longitudinal stripes of neuroblasts or glial precursors can be distinguished on each side that are the precursors of neurons and glia of the ventral nerve cord: ventral midline mesectodermal cells that separate the right and left halves of the ventral nerve cord, medial neuroblasts or glial precursors that express the NK-2 homeobox gene, and lateral neuroblasts or glial precursors that have little or no NK-2 mRNA.

The monolayer of neuroblasts that give rise to the ventral nerve cord is also divided along the anterior-posterior axis of the embryo into 14 parasegments; most parasegments consist of posterior compartment neuroblasts that

FIGURE 4. Distribution of NK-2 mRNA in *Drosophila* embryos as a function of developmental age (from Ref. 19). The RNA probe used for *in situ* hybridization was from the 3'-untranslated region of NK-2 cDNA; the probe did not contain the homeobox. (A) Expression of the NK-2 gene is initiated during stage 4, the syncytial blastoderm stage. (B) Stage 5-6, side view. (C) Ventral view, stage 6, early gastrulation. (D) Ventrolateral view in late stage 6 embryo. (E) Side view of embryo; gastrulation is almost completed. Late stage 7, about 185 minutes after fertilization. Notice the apparent segmentation of the NK-2-positive region. (F) Late stage 7 illustrating apparent segmentation of NK-2-positive region. (G) Side view stage 9 embryo 3.7-4.3 hours after fertilization. Two clusters of neuroectodermal cells and/or neuroblasts that contain NK-2 mRNA can be seen per hemisegment. (H) Side view of stage 9-10 embryo. (I) Ventral view of stage 10 embryo. Two clusters of medial neuroectodermal cells and/or neuroblasts that contain NK-2 mRNA are present per hemisegment. (J) Stage 9-10 embryo, side view.

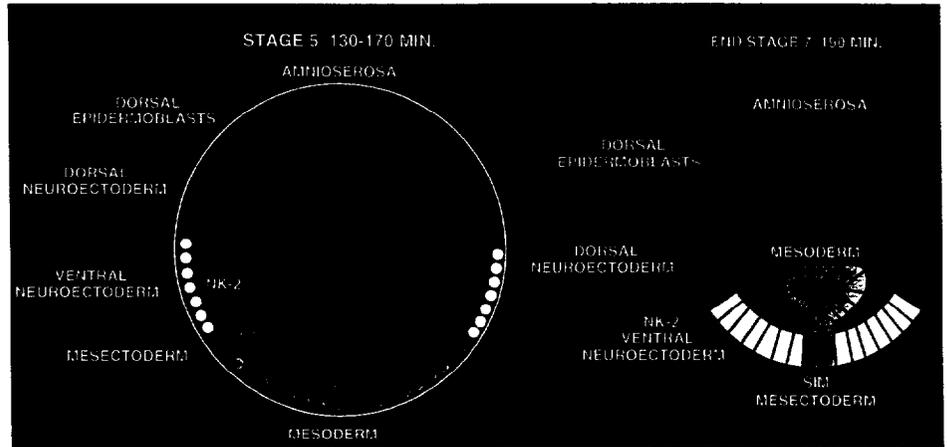


FIGURE 5. Schematic drawings of cross-sections of embryos before gastrulation (stage 5) and after gastrulation (end of stage 7) to show the ventral neuroectoderm nuclei or cells (yellow) that express the NK-2 gene.

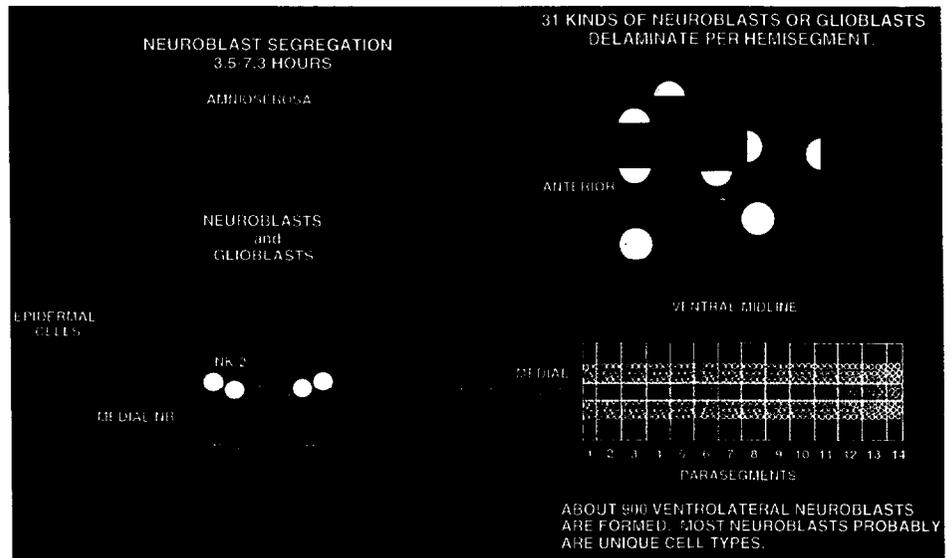


FIGURE 6. Some of the neuroectodermal cells delaminate and form a monolayer of neuroblasts and/or glioblasts immediately above the epidermal cell layer. The figure on the left is a schematic illustration of a cross-section of an embryo to show the monolayer of neuroblasts or glioblasts that delaminate from the neuroectodermal cell layer. Blue: ventral midline mesectodermal cells that express the *sim* gene. Yellow: medial neuroblasts that express the NK-2 gene. Red: lateral neuroblasts and/or glioblasts. Upper right panel shows 31 kinds of neuroblasts or glioblasts that delaminate per average hemisegment. Four of the delaminated cells have migrated to other positions; hence, only 27 delaminated cells are shown. Lower right panel: Ventral view of the monolayer of neuroblasts and/or glioblasts to illustrate ventral-dorsal and anterior-posterior patterns. The yellow medial neuroblasts and/or glioblasts express the NK-2 gene.

express the engrailed (*en*) homeobox protein^{49,50} and anterior compartment cells that do not express *en*. Hence the 31 neuroblasts or glial precursor cells that segregate per hemisegment are divided into four groups of cells, depending on the position of the cells and the expression of the NK-2 and *en* genes: medial anterior compartment cells that have high levels of NK-2 mRNA but no *en* mRNA, lateral anterior compartment cells that lack NK-2 and *en* mRNA, medial posterior compartment cells that have high levels of both NK-2 and *en* mRNA, and lateral posterior compartment cells that have *en* mRNA and low levels of NK-2 mRNA.

Neuroblasts start to divide soon after they segregate from the neuroectodermal cell layer. Each neuroblast division gives rise to a small ganglion mother cell and a large neuroblast, which becomes smaller with each division (FIG. 7). Each ganglion mother cell divides only once and gives rise to two neurons. The first neuroblasts to originate divide about eight times, whereas the last neuroblasts divide five times.⁴⁷⁻⁴⁸ Therefore, a single neuroblast may be the precursor of 10 to 16 neurons.

The neuroblasts that express the NK-2 gene⁵¹ in a thoracic segment at the end of neuroblast segregation (late stage 11) are shown schematically in FIGURE 8. It should be emphasized that the abundance of NK-2 mRNA changes dynamically during development. NK-2 is expressed by two longitudinal columns of medial neuroblasts on each side; however, the abundance of NK-2 mRNA usually is higher in the column of neuroblasts adjacent to the mesectodermal ventral midline cells than in the second column of neuroblasts. All neuroblasts in the posterior compartment express the NK-2 gene; however, the lateral neuroblasts contain much less NK-2 mRNA than do the medial neuroblasts. Hence, a medial to lateral gradient of NK-2 mRNA is established in both the anterior and posterior compartments. The amount of NK-2 mRNA in neuroblasts 2-1, 2-3, 5-1, and 5-2 (that is, immediately after or before the posterior compartment) decreases during development, resulting in the formation of two clusters of neuroblasts that express the NK-2 gene per hemisegment, one cluster in the anterior compartment and the second consisting of posterior compartment neuroblasts. Some ganglion mother cells and neurons also express the NK-2 gene; however, the levels of NK-2 mRNA decrease markedly in some cells during later stages of embryonic development. These results show that about half of the ventrolateral neuroblasts express the NK-2 gene and that medial neuroblasts contain higher levels of NK-2 mRNA than do intermediate or lateral neuroblasts.

The pattern of expression of the NK-2 gene also was determined in various mutant lines of flies as a function of developmental age.⁵¹ The NK-2 gene was found to be activated initially by dorsal in the ventral half of the embryo. However, the NK-2 gene normally is not expressed in the mesodermal anlage because of repression by *snail*, in the mesectodermal anlage because of repression by *sim*, or in part of the lateral neuroectodermal anlage or dorsal epi-

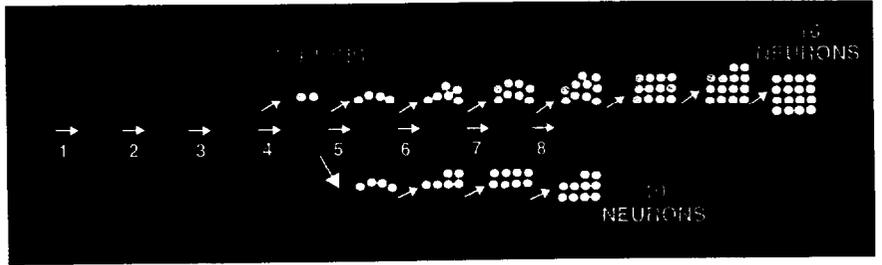


FIGURE 7.

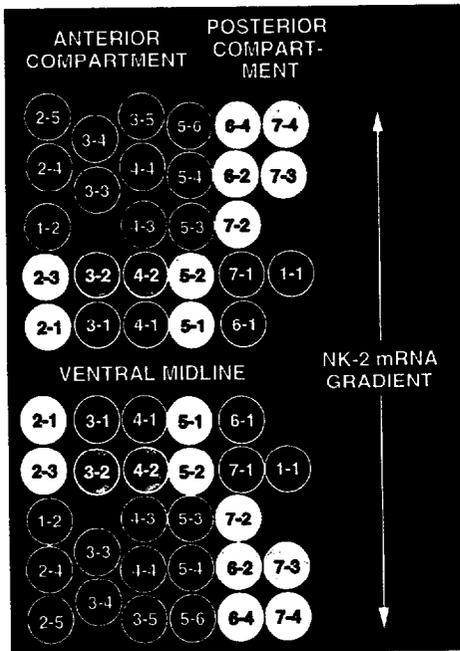


FIGURE 8.

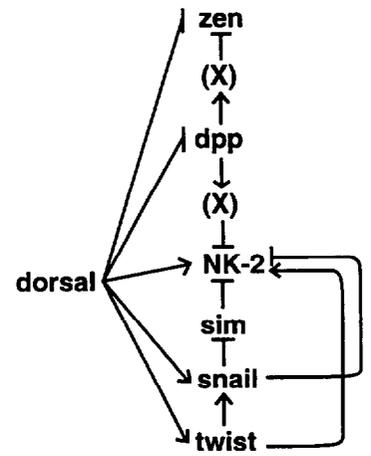


FIGURE 9.

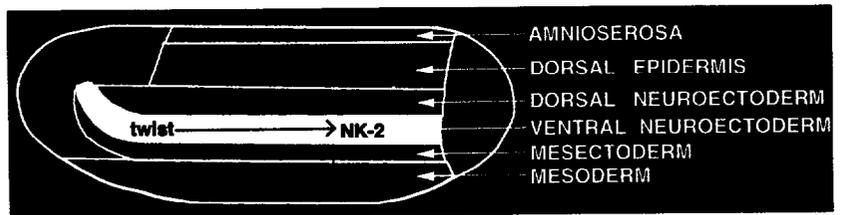


FIGURE 10.

dermal anlage because of repression mediated by *dpp*. Both dorsal and twist were found to be required to activate the NK-2 gene in the hindgut primordium and the posterior midgut primordium.

During stage 4 dorsal activates the *twist*, *snail*, and NK-2⁵¹ genes (FIG. 9). Dorsal represses *dpp* and the *zen* genes in the ventral and lateral portions of the embryo; however, the concentration of dorsal is too low for effective repression of *dpp* or *zen* genes in the dorsolateral and dorsal portions of the embryo, respectively (for reviews see Refs. 52–54). *snail* is expressed only in the most ventral nuclei, which comprise the mesodermal anlage.^{55–56} *sim* is activated in both the mesectodermal and mesodermal anlage, but *sim* is expressed only in the mesectodermal anlage because of repression by *snail*.^{34,57–60} The NK-2 gene is activated in the mesodermal, mesectodermal, ventral neuroectodermal, and part of the dorsal neuroectodermal anlagen, but is repressed by *snail* in the mesodermal anlage, by *sim* in the mesectodermal anlage, and by a gene regulator that has not thus far been identified whose repression is mediated by *dpp* in the dorsal neuroectodermal anlage.⁵¹ *twist* is expressed in the mesodermal, mesectodermal, and the ventral portion of the ventral neuroectodermal anlagen.^{61,62,35} *twist* protein activates the *snail* gene in the mesodermal primordium³⁶ and the NK-2 gene in the ventral portion of the neuroectoderm.⁵¹ *snail* represses the *sim* and NK-2 genes in the mesodermal anlage,^{51,59,60} while *sim* represses the NK-2 gene in the mesectodermal anlage.⁵¹ The hierarchical organization of gene regulation results in the appear-

FIGURE 7. Neuroblast division. A neuroblast (*green*) divides 5–8 times. Each neuroblast division is unequal and gives rise to a slightly smaller neuroblast and a much smaller ganglion mother cell (*red*). Each ganglion mother cell divides once, giving rise to two neurons (*yellow*).

FIGURE 8. A ventral view of neuroblasts in a thoracic segment at the end of stage 11 is shown using the neuroblast nomenclature of Doe.⁴⁹ Neuroblasts shown in color express the NK-2 gene (from Ref. 51). The varying darkness of the neuroblast color from *brown* to *tan* represents the relative abundance of NK-2 mRNA; for example, in order of decreasing abundance of NK-2 mRNA, we see *brown* (neuroblast 4-1), *orange* (4-2), *yellow* (5-1), *pale yellow* (5-2), and *tan* (2-1). No NK-2 mRNA was detected in black neuroblasts. Medial neuroblasts closest to the ventral midline usually contain more NK-2 mRNA than do neuroblasts in a more lateral position in that vertical row. All posterior compartment neuroblasts express the NK-2 gene. A medial to lateral NK-2 mRNA gradient is present.

FIGURE 9. Regulation of NK-2 gene expression deduced from the patterns of expression of the NK-2 gene in various mutant lines of flies (from Ref. 51). An *arrowhead* represents gene activation, while a terminal *bar* represents gene repression. (X) corresponds to an unidentified repressor mediated by *dpp*.

FIGURE 10. Side view of an embryo showing the ventral-dorsal pattern of the anlagen indicated and the hierarchically organized regulation of NK-2 gene expression.⁵¹ An *arrowhead* corresponds to gene activation, while a terminal *bar* represents repression. *dpp* indirectly mediates repression of the NK-2 gene in dorsal neuroectoderm, via (X), an unidentified repressor. The NK-2 gene is repressed by *sim* in the mesectodermal anlage and by *snail* in the mesodermal anlage.

ance of six horizontal stripes which, from ventral to dorsal, comprise the mesodermal, mesectodermal, ventral neuroectodermal, dorsal neuroectodermal, dorsoepidermal, and amnioserosa anlagen, as shown in FIGURE 10.

The ventral border of the horizontal stripe of nuclei that synthesize NK-2 mRNA is created by repression of the NK-2 gene by *snail* initially and then by *sim*, while the dorsal border of the stripe is created by a different, unidentified species of repressor mediated by *dpp*.⁵¹ Thus, the ventral and dorsal borders of the NK-2-positive stripe of nuclei are created independently by different species of repressors. The width of the NK-2-positive stripe of nuclei and the position of the stripe on the ventral-dorsal axis of the embryo are not fixed, but can be shifted by the combined effects of proteins that induce and repress the NK-2 gene. Rao, Vaessin, Jan, and Jan⁶³ have shown previously that the position of the neuroectoderm in the *Drosophila* embryo is shifted in appropriate mutants. Levine and his colleagues⁶⁴⁻⁶⁶ have shown that the leading and trailing edges of a vertical stripe of nuclei that express the *eve* gene (eve stripe 2) are formed independently by *giant* and *Krüppel*, respectively, which repress the *eve* gene. Although the gradient of inducer and the repressors are different, the formation of a horizontal stripe of nuclei that express the NK-2 gene resembles the formation of a vertical stripe of nuclei that express the *eve* gene.

Neuroectodermal cells develop at different rates and segregate as neuroblasts at different times, depending upon their position in the embryo (for review see Ref. 67). Therefore, it is likely that the expression of a proneural gene is the rate-limiting step in the development of ventral neuroectodermal cells and segregation of medial neuroblasts. Our working hypothesis is that NK-2 is a proneural gene required for the formation of medial neuroblasts. Deletion of the NK-2 gene and some neighboring genes is a homozygous lethal deficiency and results in embryos with grossly defective ventral nerve cords that lack many neurons compared to wild-type embryos.⁶⁸ At the present time we are trying to obtain specific mutations of the NK-2 gene.

Initially all nuclei in the ventrolateral neurogenic anlage are committed to the neuroblast pathway of development. However, only about 25% of the neuroectodermal cells segregate as neuroblasts; most of the remaining neuroectodermal cells become ventrolateral epidermoblasts. Campos-Ortega and others have identified a set of neurogenic genes, *Notch*, *Delta*, *almondex*, *big brain*, *master mind*, *neuralized*, and the *Enhancer of split* [*E(spl)*] complex of genes, whose expression is required to turn off the neuroblast pathway of development and/or turn on the epidermoblast pathway of development (for reviews see Refs. 67, 69, and 70). *Delta* and *Notch* encode cell membrane proteins that interact with one another and contain multiple EGF repeats⁷¹⁻⁷⁵ (*Delta* and *Notch* proteins are thought to function as a ligand and corresponding receptor). The *E(spl)* complex of genes contains a cluster of related genes that encode similar basic helix-loop-helix DNA binding proteins (HLH-

m3, HLH-m5, HLH-m7, HLH-m8 [E(spl)], HLH-m β , HLH-m γ , and HLH-m δ , which are thought to be required for epidermoblast development,⁷⁶⁻⁷⁹ although some redundancy of HLH proteins is likely. The available information suggests that direct contact between a segregated neuroblast and neighboring neuroectodermal cells turns off the neural pathway of development and activates the epidermoblast pathway of development in the neuroectodermal cells, a process termed lateral inhibition. The switch from neuroectodermal to epidermoblast pathway of development is blocked by mutation of a neurogenic gene (or by deletion of the *E(spl)* complex of genes resulting in overproduction of neuroblasts and underproduction of epidermoblasts). Mellerick and Nirenberg⁸⁰ have shown that a null mutation of the *Delta* gene or deletion of the *E(spl)* gene complex results in overproduction of neuroblasts that express the NK-2 gene. These results show that neuroectodermal cells that express the NK-2 gene are sensitive to lateral inhibition and suggest that one or more of the E(spl) HLH proteins repress the NK-2 gene. Delta probably represses the NK-2 gene indirectly by signalling activation of *E(spl)* HLH genes. One possibility that remains to be explored is that repression of the NK-2 gene by E(spl) HLH proteins may extinguish an NK-2-dependent pathway for medial neuroblast development and activate the epidermal pathway of development.

The NK-2 homeodomain was expressed in *E. coli* and purified to essential homogeneity. Binding studies to oligodeoxynucleotides showed that the consensus nucleotide sequence for NK-2 homeodomain binding is TNAAGTGG, and that the K_D is approximately 2×10^{-10} M.⁸¹ Twenty high-affinity and additional lower-affinity NK-2 homeodomain binding sites were found in 2.2 kb of the 5'-upstream region of the NK-2 gene,⁸¹ which suggests that NK-2 protein may be required to maintain NK-2 gene expression.

Little is known about the functions of the NK-2 homeodomain protein. Our working hypothesis is that NK-2 is a proneural gene that may be required for the development of a subset of neuroblasts.

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