Development 120, 1517-1524 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

The *ventral nervous system defective* gene controls proneural gene expression at two distinct steps during neuroblast formation in *Drosophila*

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SUMMARY

Within the *Drosophila* embryo, the formation of many neuroblasts depends on the functions of the proneural genes of the *achaete-scute* complex (AS-C): *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*), and the gene *ventral nervous system defective* (*vnd*). Here, we show that *vnd* controls neuroblast formation, in part, through its regulation of the proneural genes of the AS-C. *vnd* is absolutely required to activate *ac*, *sc* and *l'sc* gene expression in proneural clusters in specific domains along the medial column of the earliest arising neuroblasts. Using *ac-lacZ* reporter constructs, we determined that *vnd* controls proneural gene expression at two distinct steps during neuroblast formation through separable regulatory regions. First, *vnd* is required to activate proneural cluster formation within the medial column of every other neuroblast row through regulatory elements located 3′ to *ac*; second, through a 5′ regulatory region, *vnd* functions to increase or maintain proneural gene expression in the cell within the proneural cluster that normally becomes the neuroblast. By following neuroblast segregation in *vnd* mutant embryos, we show that the neuroectoderm forms normally and that the defects in neuroblast formation are specific to particular proneural clusters.

Key words: *Drosophila*, neurogenesis, proneural genes, neuroblast segregation

INTRODUCTION

The cellular complexity of the central nervous system (CNS) of the *Drosophila* embryo arises as a result of the generation, specification and asymmetric division of neuroblasts (NBs; reviewed by Campos-Ortega, 1993). Neuroblasts (NBs) are singled out from equivalence groups termed proneural cell clusters within the ectoderm and then delaminate into the interior of the embryo. Concomitant with the process of NB delamination, spatial cues apparently bestow unique identities upon individual NBs (Chu-LaGraff and Doe, 1993). NBs segregate in an invariant spatiotemporal sequence, consisting of five waves (SI-SV; Campos-Ortega and Hartenstein, 1985; Doe, 1992), to form a rigidly stereotyped sub-epidermal pattern of neural stem cells. After delaminating, NBs divide repeatedly to create the neurons and glia that make up the larval CNS.

The process of NB formation depends on the functions of the proneural genes of the AS-C and the gene *vnd* (Jimenez and Campos-Ortega, 1979, 1987; White, 1980; Ghysen and Dambly-Chaudiere, 1988). Removal of the genetic function of either the AS-C or *vnd* results in the loss of roughly 25% of all segmental NBs (Jimenez and Campos-Ortega, 1990). Since embryos doubly mutant for the AS-C and *vnd* lack roughly 50% of all segmental NBs, it has been suggested that the AS-C and *vnd* control the formation of non-overlapping sets of NBs (Jimenez and Campos-Ortega, 1990).

A large body of work has illuminated the developmental regulatory mechanisms that control both the expression of the proneural genes of the AS-C and NB formation (reviewed by Ghysen and Dambly-Chaudiere, 1988; Campos-Ortega and Jan, 1991 Simpson, 1990; Artavanis-Tsakonas and Simpson, 1991; Jimenez and Modolell, 1993). *ac*, *sc* and *l'sc* each encode basic helix-loop-helix type transcription factors (Villares and Cabrera, 1987; Alonso and Cabrera, 1988; Martin-Bermudo et al., 1993) and are initially expressed within the primordium of the embryonic CNS, the neuroectoderm, in a reproducible array of cell clusters, termed 'proneural clusters', from which single NBs later arise (Cabrera et al., 1987; Romani et al., 1987; Martin-Bermudo et al., 1991; Skeath et al., 1992). Expression of one or more of these genes within the cells of a cluster appears to bestow on these cells a general neural competency. A 'proneural cluster' constitutes an equivalence group where all cells can, although only one cell will, become a NB. The generation of the initial clustered pattern of proneural gene expression is governed by the combined action of the gene products of the pair-rule and dorsal-ventral patterning genes. These proteins presumably act through a vast array of region specific enhancers found within the AS-C to carve out clusters of *ac*, *sc* or *l'sc* gene expression at precise and reproducible coordinates within the *Drosophila* embryo (Martin-Bermudo et al., 1991 and 1993; Skeath et al., 1992). Within each cluster, the cell that comes to express the proneural gene(s) to the highest level is favored to become the NB or neural stem cell (Cubas et al., 1991; Ruiz-Gomez and Ghysen, 1993). Once chosen, the NB, which retains proneural gene expression, initiates a cell-communication pathway

(lateral inhibition) mediated by the gene products of the neurogenic genes. The process of lateral inhibition removes proneural gene expression and, hence, neural competency from the remaining cells of the cluster (Lehmann et al., 1983; Cabrera, 1990; Skeath and Carroll, 1992; reviewed by Campos-Ortega, 1993 and Simpson, 1990).

Despite the detailed knowledge of the regulation of and the roles played by the *ac*, *sc* and *l'sc* genes during NB formation, little is known about the influence *vnd* has on this process, except that it is required for 25% of all NBs to segregate (Jimenez and Campos-Ortega, 1990). For example, is *vnd* directly involved in neurogenesis or does it influence the nervous system by affecting some other aspect of development? If direct, does *vnd* act in parallel with or in tandem with *ac*, *sc* or *l'sc* to create proneural clusters? Does it act at a later step to specify which cell within each proneural cluster becomes the NB, or like *ac*, *sc* or *l'sc* does it function more than once during the process of NB formation? In order to begin to elucidate the role *vnd* plays during the initial steps of embryonic neurogenesis, we determined its effect on the initial pattern of *ac*, *sc*, and *l'sc* proneural clusters and of the ten SI NBs that segregate from these proneural clusters. To our surprise, we found that *vnd* is required for the formation of *ac*, *sc*, and *l'sc* proneural clusters and is specific to the medial column of every other NB row. We used two different *ac* reporter constructs to determine that *vnd* controls proneural gene expression at two distinct steps during NB formation through separate regulatory regions. First, *vnd* acts, directly or indirectly, through regulatory regions 3′ to *ac* to activate gene expression in proneural clusters within the medial column; then, through regulatory regions 5′ to *ac*, *vnd* functions either to heighten proneural gene expression in the cell that becomes the NB or to maintain proneural gene expression in this cell. By following NB segregation in embryos mutant for *vnd* we show that the effect of *vnd* on SI NB segregation parallels its effect on proneural cluster formation.

MATERIALS AND METHODS

Fly strains

The following fly strains were used: B40 (Martinez and Modolell, 1991) and 101H10 (Skeath et al., 1992) *ac-lacZ* reporter transformant lines, vnd^{6}, *Df(1)svr*; $Df(1)y^{3PL}sc^{8R}$, $Df(1)sc^{B57}$, *FM7-ftzlacC* balancer stock; Oregon R was the wild-type strain used (See Lindsley and Zimm (1992) for descriptions of mutant stocks). These strains were obtained from the labs of Juan Modolell, Peter Gergen, Kalpana White, Michael Young, Y.N and L.Y. Jan and from the Bloomington Stock Center.

In situ hybridization and immunochemistry

ac, *sc*, *l'sc* and *lacZ* transcripts were detected in appropriately staged embryos by hybridization with digoxigenin-labeled antisense RNA probes specific for each transcript made with the Genius 4 kit (Boehringer-Mannheim) following the protocol of Jiang et al. (1991). Double-antibody labeling experiments were carried out as described by Skeath et al. (1992). For detection of *sna* protein two monoclonal antibodies 1SN G5 and 2SN 5H (kindly provided by Audrey Alberga; Mauhin et al., 1993) were used together, each at a 1:10 dilution; for detection of *en* expression the INV4D9 monoclonal antibody (kindly provided by Nipam Patel, Carnegie Institute of Embryology, Baltimore, MD) was used at a 1:10 dilution. For detection of *hb* protein expression a polyclonal antiserum (kindly provided by James Langeland, University of Wisconsin-Madison) was used at a dilution of 1:400. For detection of β-galactosidase, a polyclonal antiserum was used at a dilution of 1:1000. In the experiments where *ac*, *sc* and *l'sc* transcript and *sna* protein expression were examined in *vnd* mutant embryos, an *FM7-ftzlacC* blue balancer was used to identify unambiguously mutant embryos. In order to analyze 101H10 reporter gene expression in the absence of *vnd* function, we crossed a stock carrying 4 copies (this stock contains homozygous viable inserts on the second and third chromosomes) of the 101H10 construct to *Df(1)svr* females. Virgin females from this cross were then mated to males carrying four copies of the 101H10 construct and the 101H10 expression pattern was examined in their progeny. Mutant embryos were identified by co-labeling with *l'sc* and *lacZ* antisense probes; those embryos that did not express *l'sc* were of the *Df(1)svr* genotype. B40 expression in *vnd* mutant embryos was determined by crossing virgin females heterozygous for *vnd* to B40/*CyO* males and analyzing *lacZ* expression in the resulting embryos. Approximately one quarter of these embryos exhibited the loss of *lacZ* gene expression within the medial column.

RESULTS

Overview of proneural gene expression and SI and SII NB segregation

Our work has focused on elucidating the genetic regulatory mechanisms that govern proneural gene expression during early neurogenesis in the *Drosophila* embryo. In this report, we focus on the role of the *vnd* gene in regulating the expression patterns of the proneural genes *ac*, *sc* and *l'sc* during SI NB segregation in the *Drosophila* embryo. We will first summarize briefly the patterns of *ac*, *sc* and *l'sc* gene expression during, and the pattern of NBs generated by, the first two waves of NB segregation. Previous studies have mapped precisely the clustered expression of *ac*, *sc* and *l'sc* prior to and concomitant with SI NB segregation (Martin-Bermudo et al., 1991; Skeath and Carroll, 1992; Skeath et al., 1992). The registration of proneural gene expression in the neuroectoderm prior to SI NB segregation is best described using an orthogonal grid of 12 squares (see Fig. 1A; left). Before SI NB segregation, *ac* and *sc* are co-expressed in four cell clusters (roughly 5-7 cells are found in each cluster) per hemisegment - the medial and lateral clusters of rows B and D (Fig. 1A; left and Fig 2A); whereas *l'sc* is expressed in a circular pattern of eight adjoining proneural clusters - the medial, intermediate and lateral clusters of rows A and C and the medial and lateral clusters of row D (Fig. 1A; left and Fig. 2B). *l'sc* expression comes on slightly later in the intermediate cluster of row A than in all other SI clusters; in the embryo shown in Fig. 2B *l'sc* has yet to be activated in this cluster. Within every cluster proneural gene expression is quickly restricted to a single cell, the presumptive NB. The registration of proneural gene expression is retained by the NBs that segregate from each cluster (Fig. 1A; right) with one exception: the lateral NB of row B (NB3-5) expresses *l'sc* even though the cluster from which it arises does not (Martin-Bermudo et al., 1991).

Shortly after the SI NBs (Fig. 1B; left) have formed, a second wave of NB segregation occurs (SII; Campos-Ortega and Hartenstein, 1985; Doe, 1992). In contrast to SI NBs, which form predominantly in the medial and lateral columns (Fig. 1B; left), the majority of SII NBs form within the intermediate column (Fig. 1B; right). SII NBs arise from proneural clusters expressing only the *l'sc* gene (Martin-Bermudo et al.,

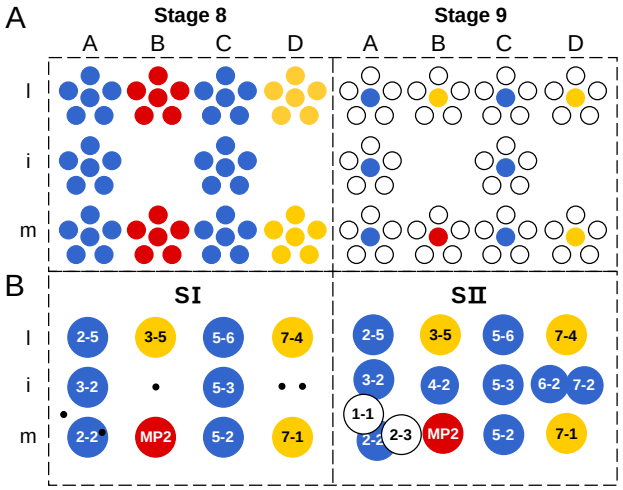

Fig. 1. Schematics of proneural gene expression before and during SI NB segregation and the pattern of SI and SII NBs. (A) *ac*, *sc* and *l'sc* gene expression in two consecutive wild-type hemisegments at stage 8 (left) when the proneural genes are expressed in cell clusters and at stage 9 (right) after expression has been restricted to single cells, the presumptive SI NBs. Each cluster of six cells expressing *l'sc* (blue), *ac* and *sc* (red) *ac*, *sc* and *l'sc* (yellow) represents a separate proneural cluster from which a single NB arises. During stage 8 proneural gene expression initially divides each hemisegment into a grid consisting of four transverse rows (A-D) and three longitudinal columns (medial, m; intermediate, i; and lateral, l). *ac* and *sc* are expressed in the medial and lateral clusters of rows B and D; *l'sc* in the medial, intermediate and lateral clusters of rows A and C and the medial and lateral clusters of row D. The registration of proneural gene expression is largely conserved in the NB pattern with the exception that the lateral NB of row B (NB 3-5), but not the cluster from which it arises, expresses *l'sc* . (B) The pattern of SI and SII NBs (NBs colored as above; *l'sc* may be expressed in NBs 1-1 and 2-3). Ten NBs segregate during SI (stage 8) NB segregation (left; NBs numbered as per Doe, 1992 and colored as above); eight are found in medial and lateral columns (left). In contrast, most SII NBs (stage 9) form in the intermediate column (right). Black dots in left panel indicate where SII NBs will form. 15 NBs occupy each hemisegment after SII NB segregation. Three subsequent waves of NB segregation (SIII-V) produce the final pattern of 30 NBs per hemisegment (Doe, 1992). Anterior: left.

1991). The prior segregation of the SI NBs allows one to predict unambiguously where SII NBs will form within the NB array. For example, the SI NBs MP2, 3-5, 3-2 and 5-3 encircle the location where the SII NB 4-2 will arise (Fig. 1B; right; Fig. 4).

vnd controls proneural cluster formation within highly specific AP and DV domains

Although the AS-C and *vnd* are thought to control the formation of largely complementary sets of NBs, the SI NB MP2 does not form in the absence of either the AS-C or *vnd* (Jimenez and Campos-Ortega, 1990). This suggested to us that the genes of the AS-C and *vnd* may interact to control the formation of this and other NBs. To investigate this possibility, we determined the expression patterns of the *ac*, *sc* and *l'sc* transcripts during SI NB segregation in *vnd* mutant embryos. *vnd* was found to be required for each transcript to be expressed within the proneural clusters of the medial column of rows B and D (Fig. 2; data not shown for *sc*). The loss of *vnd* gene function had very little effect on proneural gene expression medially in rows A or C and no effect on gene expression in the lateral column (Fig. 2C,D). Since both *ac* and *sc* are normally expressed only in the medial and lateral columns of rows B and D (Fig. 2A), there is a complete absence of *ac/sc* expression in the medial column in embryos mutant for *vnd* (Fig. 2C); no change to *ac/sc* expression is observed within the lateral column. Similarly, in *vnd* mutant embryos, *l'sc* expression is lost from the medial, but not the lateral, column of row D (inset to Fig. 2D). *l'sc* is, however, still expressed in a normal pattern in rows A and C (compare Fig. 2C and 2D), although its appearance within these rows is delayed slightly. The apparent absence of *l'sc* expression in the lateral column of row D in Fig. 2D is due to the slightly older age of this embryo in relation to the one shown in the inset. *l'sc* expression normally disappears from row D before it does from rows A or C. The normal pattern of *l'sc* expression in rows A and C, the normal appearance of the ventral midline and the formation of most of the medial and intermediate SI NBs of rows A and C (see below) in *vnd* mutant embryos suggest that *vnd* is not required within the medial column for cell viability or fate specification. Rather, the specific absence of proneural clusters from the medial column of rows B and D indicates that *vnd* is required along precise domains of both the anterior-posterior (AP) and dorsal-ventral (DV) axes to activate proneural cluster formation.

vnd activates proneural gene expression through separate regulatory regions at two distinct levels: proneural cluster formation and NB segregation

In order to define more precisely the influence of *vnd* on proneural gene expression, we determined the expression pattern of two *ac-lacZ* reporter constructs in embryos mutant for *vnd*. These two reporter constructs contain either 10 kb of DNA immediately 3′ to ac (101H10; Skeath et al., 1992) or 3.8 kb of DNA immediately 5′ to *ac* (B40; Martinez and Modolell, 1991). Within the embryonic CNS, 101H10 drives gene expression in row B, and to a lesser extent, in row D proneural clusters (Fig. 3A; Skeath et al., 1992); while B40 initially directs gene expression within the medial and lateral column of row B, generally in single cells - the presumptive row B SI NBs - before or during NB segregation (Fig 3C; Panganiban, G., Skeath, J. B. and Carroll, S. B., unpublished data; data not shown). The expression of both constructs in the embryonic CNS is independent of AS-C function (Panganiban, G., Skeath, J. B. and Carroll, S. B., unpublished data). Thus, any alterations to the patterns driven by these constructs in embryos mutant for *vnd* cannot be attributed to the loss of proneural gene expression that occurs in this background.

In *vnd* mutant embryos, the expression patterns driven by each construct are altered. In the case of the 3′ 101H10 reporter gene fusion, *lac-Z* expression in the medial proneural clusters is almost completely abolished; a very low level of striped *lacZ* expression can still be observed that connects the lateral clusters of rows B and D in these embryos (Fig. 3B). Similarly, 5′ B40 reporter gene expression is absent from the medial, but not the lateral presumptive NBs of row B (Fig. 3D). This is not due simply to the failure of these NBs to form because the expression of B40 is not obligately coupled to NB formation (Panganiban, G., Skeath, J. B. and Carroll, S. B., unpublished data). In the absence of the entire AS-C ($Df(1)sc^{B57}$), the

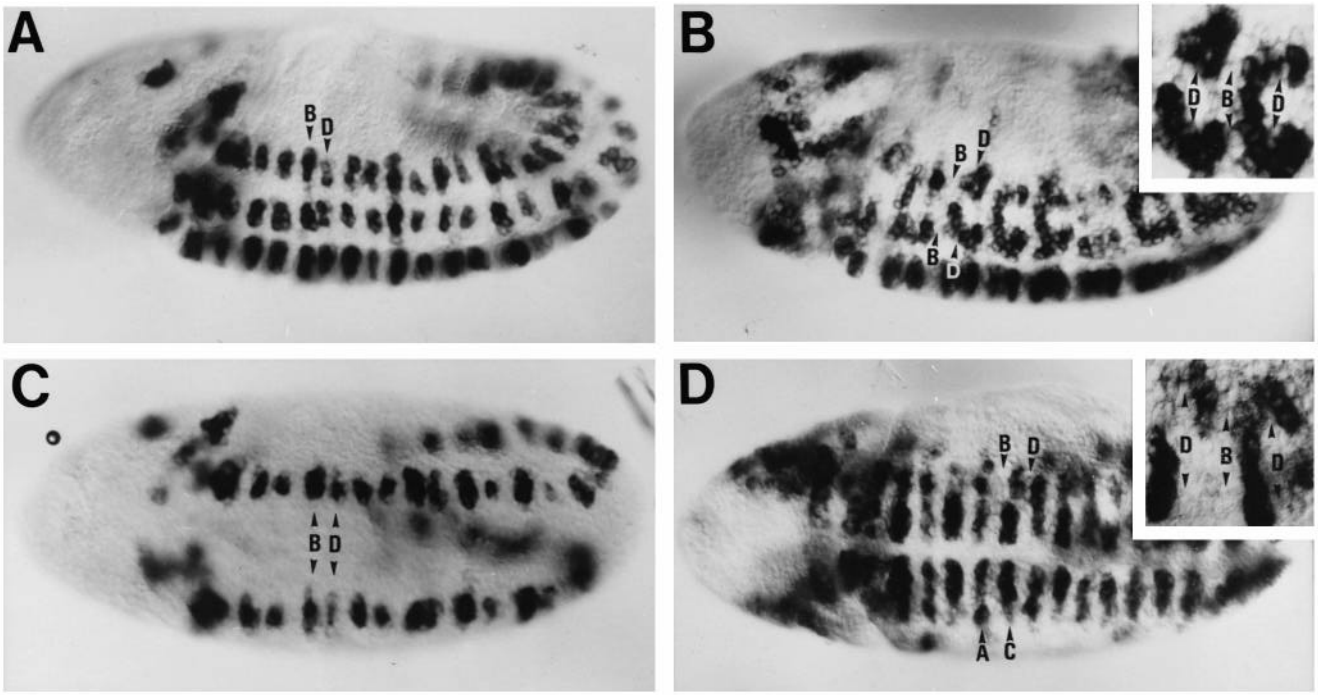

Fig. 2. *vnd* controls medial proneural cluster formation within rows B and D. Ventrolateral and ventral views of *ac* (A,C) and *l'sc* (B,D) RNA expression in mid-late stage 8 wild-type (A,B) or *vnd* mutant (C,D) embryos. In wild-type embryos, *ac* is expressed both medially and laterally in rows B and D (A). In *vnd* mutant embryos *ac* expression is completely abolished within the medial column of rows B and D (C). *l'sc* is normally expressed in the medial and lateral columns of rows A, C and D and in the intermediate column of rows A and C (B and inset). In *vnd* mutant embryos, *l'sc* gene expression is lost from the medial column of row D (inset to D). Insets to B and D show high magnification views of two hemisegments labeled for *l'sc* RNA in wild type (C) or *vnd* mutant (D) embryos. The inset to B is from the same embryo depicted in B; while the inset to D is from a younger (early stage 8 embryo) than shown in D and illustrates that in *vnd* mutant embryos *l'sc* is activated laterally but not medially within row D. Although in *vnd* mutant embryos there is a delay in the expression of *l'sc* within row A and to a lesser extent row C (inset to D), *l'sc* does eventually realize its normal pattern and level of expression within these rows (D). In D, the apparent absence of *l'sc* expression in the lateral column of row D is due to the age of the embryo; *l'sc* expression in row D normally disappears before that in rows A or C. Anterior: left.

medial SI NB of row B (MP2) hardly ever forms (2.5%; *n*=38 hemisegments; see also Jimenez and Campos-Ortega, 1990); yet, in $Df(1)sc^{B57}$ embryos B40 drives gene expression in the cells that would normally become MP2 (Panganiban, G., Skeath, J. B. and Carroll, S. B., unpublished data). These results show that *vnd* controls *ac* (and most likely proneural gene expression in general) at two distinct levels. First, through regulatory regions 3′ to *ac*, *vnd* directly, indirectly or cooperatively activates *ac* gene expression within the medial proneural clusters of rows B and D. Then, through regulatory regions 5′ to *ac*, *vnd* appears to increase or maintain proneural gene expression in the cell that becomes the NB. As the level of proneural gene activity appears to be a key determinant of which cell in a cluster becomes the NB, *vnd* may be involved in singling out which cell within the cluster becomes the NB.

vnd and the genes of the AS-C control the formation of overlapping sets of SI NBs

The dramatic alterations to proneural gene expression in *vnd* mutant embryos led us to determine the effects of removing *vnd* or AS-C gene function on SI NB formation. Such experiments have been performed previously (Jimenez and Campos-Ortega, 1990), however, the presence or absence of specific SI NBs was not assayed. To follow SI NB segregation in *vnd* and AS-C mutant embryos, we used monoclonal antibodies generated against the *snail* protein product (generously provided by Audrey Alberga; Mauhin et al., 1993). The *snail* gene is expressed in all NBs as they arise within the developing CNS (Fig. 4B; Alberga et al., 1991; Kosman et al., 1991). Identical results were obtained using an antibody directed against the *hunchback* (*hb*) protein product (J. B. S. data not shown) which also marks all NBs (Jimenez and Campos-Ortega, 1990). In *vnd* mutant embryos, the medial SI NBs of rows B and D (MP2 and NB 7-1) do not form (Fig. 4G, arrows); whereas all lateral NBs form normally (Fig. 4G). At a low frequency (<25%; *n*>70 hemisegments) NBs from the medial and intermediate columns of rows A and C (NB 2-2, 3-2, 5-2, and 5-3) do not arise. This variable loss of NBs from rows A and C may result from the observed delay of *l'sc* gene expression in these rows or from an effect of removing *vnd* function that is independent of AS-C function. In embryos carrying the deficiency $Df(1)y^{3PL}sc^{8R}$, there is no proneural gene expression within the proneural clusters of row B (Martin-Bermudo et al., 1991; Skeath et al., 1992) and the medial NB (MP2) of row B forms roughly 14% of the time (79 hemisegments scored), while the lateral NB (NB 3-5) forms less than 50% of the time (79 hemisegments scored; arrowheads, Fig. 4F); all other SI NBs form normally. In the complete absence of the AS-C, the medial NB of row B (MP2)

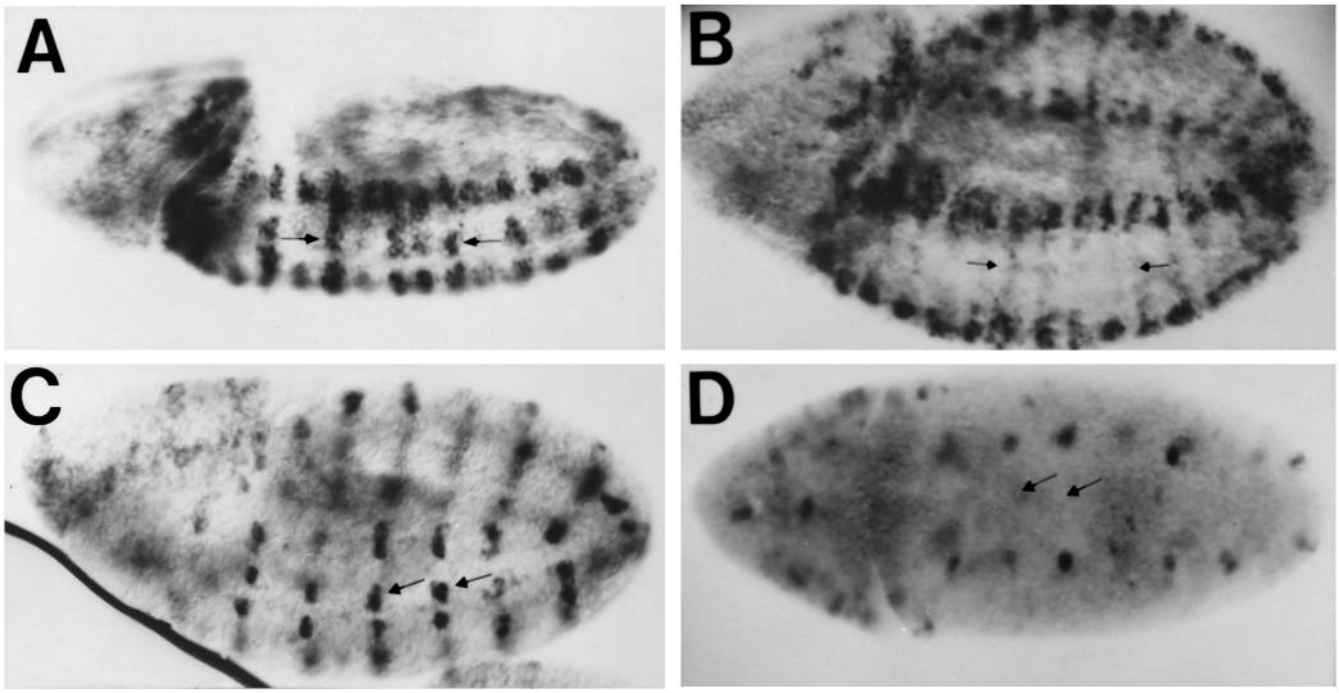

Fig. 3. *vnd* controls both proneural cluster and NB formation through separate regulatory regions. Ventrolateral and ventral views of wild type (A,C), *Df(1)svr* (B) and *vnd6* (D) mutant embryos carrying either the 101H10 (A,B) or B40 (C,D) *ac-lacZ* reporter constructs. The 101H10 construct contains 10 kb of DNA immediately 3′ to *ac*; this construct drives *lacZ* gene expression in a pattern of cell clusters that closely approximates to the initial pattern of *ac* (compare A with Fig. 2A). B40 contains the *ac* promoter and 3.8 kb of immediately 5′ DNA and drives *lacZ* expression in single, or occasionally two cells, the future medial (arrows) and lateral NBs of row B (C). In a *Df(1)svr* mutant background (this deletion removes the AS-C, the loss of which has no effect on 101H10 expression, and *vnd*) 101H10 still drives normal expression laterally but expression is greatly reduced in the medial clusters (arrows, B). Faint *lacZ* expression can be observed in stripes of cells that join the bilaterally symmetric lateral clusters (arrows, B). Similarly, in *vnd* mutant embryos, B40 no longer drives gene expression in medial cells (arrows; D) but it still drives reporter gene expression in the lateral column (D). Anterior: left.

rarely forms and that of row D (NB 7-1) forms less than 10% of the time (*n*=38 hemisegments).

The relationship of these wild-type and mutant NB patterns to proneural cluster formation is illustrated by inspection of the dynamics of *sna* protein expression during NB segregation. In wild-type embryos, the antibody initially marks all neuroectodermal cells (Fig. 4A), but only NBs remain labeled (Fig. 4B). This is best seen during SII NB segregation. The SII NB 4-2 arises from a cell cluster located between the SI NBs MP2, 3-5, 3-2 and 5-3 (Doe, 1992; Chu-Lagraff and Doe, 1993; Fig. 1B). Before NB 4-2 segregates, a cluster of '*sna*' positive neuroectodermal cells is clearly visible above the position where NB 4-2 will form. These cells are in the ectodermal (Fig. 4C), not the NB (Fig. 4D), cell layer. Once NB 4-2 has formed, '*sna*' expression is restricted to this cell (Fig. 4E). *sna* protein is therefore expressed in proneural clusters and follows identical expression dynamics as the *ac*, *sc* and *l'sc* genes. Importantly, these *sna/hb* 'proneural clusters' arise normally in the absence of AS-C and/or *vnd* function. For example, in $Df(1)y^{3PL}sc^{8R}$ and *vnd* (Fig. 4F, arrowheads; Fig. 4G arrows) embryos, *sna* positive 'proneural clusters' are found in the neuroectodermal cells of the medial column of rows B and D and the medial and lateral columns of row B, respectively, even though in these backgrounds NBs rarely delaminate from these positions. This demonstrates that the neuroectodermal expression of *sna/hb* arises normally in these mutants but the subsequent restriction of their expression to just the NB requires AS-C and *vnd* function. The generalized neuroectodermal expression of the *sna/hb* marker proteins in the absence of AS-C or *vnd* function may reflect a general neural competency of these cells that normally requires the function of the AS-C and *vnd* genes to be realized.

DISCUSSION

We have focused on the role of the gene *vnd* during NB formation in the *Drosophila* embryonic CNS. We have shown that *vnd* is required to activate the expression of the *ac*, *sc* and *l'sc* genes in proneural clusters in precise AP (rows B and D) and DV (medial column) domains. *vnd* controls proneural gene expression at two distinct steps during neuroblast formation through separable regulatory regions (Fig. 5): (i) *vnd* activates proneural cluster formation through regulatory regions 3′ to *ac*; and (ii) *vnd* apparently increases proneural gene expression within the cell that becomes the NB through regulatory regions 5′ to *ac*. Finally, we demonstrated that the effect of removing *vnd* function on proneural cluster formation is paralleled by its effect on NB formation - SI NBs from the medial column of rows B and D do not form.

vnd controls proneural cluster formation and the level of proneural gene expression in the presumptive NB

We have shown that *vnd* is required for proneural cluster

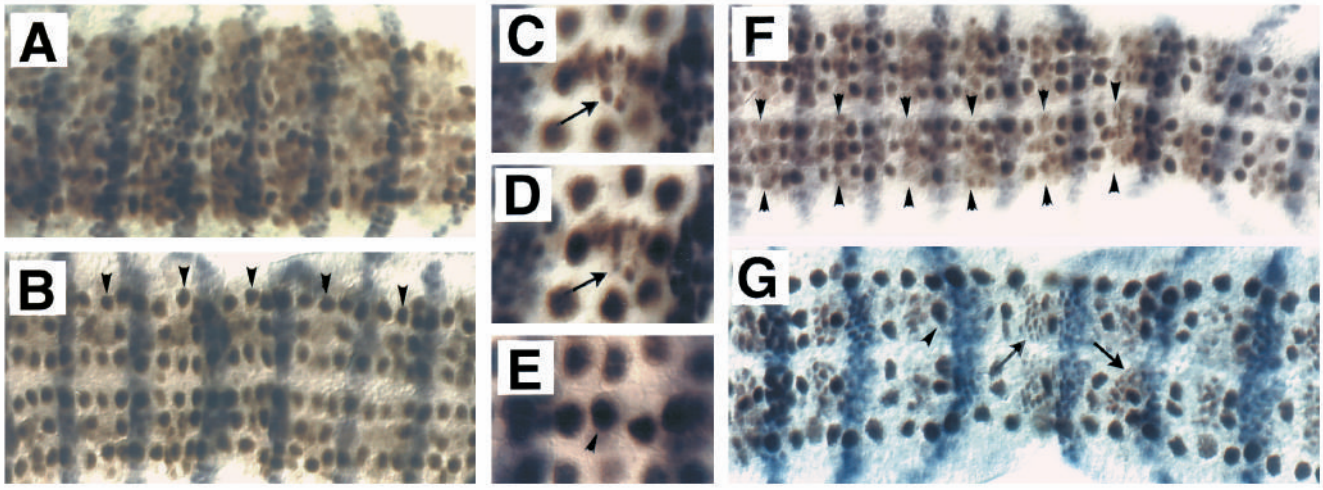

Fig. 4. NB segregation in wild type, AS-C^{-}, and *vnd* mutant embryos. Ventral views of early stage 8 and late stage 8/early stage 9 wild-type and mutant embryos co-labeled with monoclonal antibodies generated against the *sna* (brown) and *engrailed* (*en*; blue/black) proteins. *en* expression marks the posterior compartment (row D) of each segment; anterior: left. (A) wild-type pattern of *sna* protein expression in the neuroectoderm during early stage 8 at the onset of SI NB segregation and (B) late stage 8 after SI NBs have segregated. (C,D) High magnification views of *sna* protein expression in a single hemisegment about to undergo SII NB segregation (late stage 8; C, ectodermal cell layer; D, NB cell layer) and (E) in a single hemisegment that has undergone SII NB segregation. *sna* protein expression in (F) late stage 8 *Df(1)y*^{3PL}*sc*^{8R} and (G) *vnd*^{6} mutant embryos. Initially, the antigens detected by the *sna* antibody are expressed in all cells of the neuroectoderm (A); however, as the SI NBs segregate, expression intensifies within the delaminated NBs and is lost from the other cells (B). Prior to SII NB segregation new clusters of '*sna*' positive ectodermal cells arise above the position where SII NBs will delaminate (C-E). For example, prior to the formation of NB 4-2 a cluster of six '*sna*' positive cells is found above where NB 4-2 will form (arrow; C,D). These cells reside in the ectodermal (C) but not the NB (D) cell layer. Once formed, expression intensifies within NB 4-2 (arrowhead; E) and is lost from the other cells (E). In *Df(1)y*^{3PL}*sc*^{8R} mutant embryos the medial and lateral NBs of row B (MP2 and NB 3-5) fail to form the majority of the time (arrowheads; F) even though the neuroectodermal cells in row B express the *sna* antigen. In *vnd* mutant embryos the medial SI NBs of rows B (MP2) and D (NB 7-1) do not form (arrows; G); although normal appearing clusters of '*sna*' positive neuroectodermal cells are found in the regions from which these NBs arise (arrows; G). The pattern of lateral NBs is normal in *vnd* mutant embryos, however, rarely the intermediate or medial SI NBs of rows A or C fail to form (arrowhead; G).

formation during SI NB segregation in the medial column of rows B and D, but not in rows A or C, or in the intermediate or lateral columns (Fig. 5). That *vnd* is required for proneural cluster formation at all was surprising on two accounts. First, it had been suggested that the genetic activities of *vnd* and of the AS-C were required for the formation of non-overlapping sets of NBs (Jimenez and Campos-Ortega, 1990); a regulatory dependence of AS-C expression on *vnd* gene function was not expected. Secondly, the generation of the initial pattern of *ac* and *sc* proneural clusters can be explained on the basis of the combinatorial action of the gene products of the pair-rule and DV genes on the *ac* and *sc* genes (Skeath et al., 1992). That is, it did not seem necessary to invoke the existence of other genes to explain how the initial pattern of proneural clusters arises within the *Drosophila* embryonic CNS. The spatial specificity (medial column rows B and D) of *vnd* action on proneural cluster formation clearly shows that *vnd* does regulate, directly or indirectly, the initial activation of proneural clusters.

In addition to its role in proneural cluster formation, *vnd* may also help single out the cell within the cluster that becomes the NB (Fig. 5). This hypothesis arises from the properties that the B40 reporter construct displays in wild type, AS-C, and *vnd* mutant embryos. Initially, the B40 construct is activated during late stage 8 at low levels generally in single or, occasionally, two cells, within the ectodermal cell layer of the medial and lateral columns of row B (Panganiban et al., 1994). In each location, this cell, or one of the two cells, delaminates and enlarges to become a row B SI NB. B40 appears to be active in the cell that will become the NB before it segregates. Furthermore, expression of B40 within the cells normally destined to become the row B NBs does not require that these cells become NBs. For example, in embryos that harbor a deletion for the entire AS-C, the medial SI NBs of row B rarely ever form. However, in this background B40 drives gene expression within the cells that would normally give rise to these NBs (Panganiban et al., in preparation). Thus, in the absence of NB formation, the cells that would normally become the medial NBs of row B are still singled out, even though they appear to require AS-C activity to become NBs.

From the above described behavior of the B40 reporter construct, we can conclude that there is an AS-C independent process that acts prior to, or concomitant with, the process of NB commitment through regulatory regions 5′ of *ac* to increase selectively *ac* gene expression within the cells that become the medial NBs of row B. Since the cell within the proneural cluster that has the highest level of proneural gene activity is favored to become the NB (Cubas et al., 1991), then the factor(s) that activates B40/*ac* gene expression within the presumptive NBs of row B may be involved in singling out which cell becomes the NB. We have shown that the loss of *vnd* gene function abolishes B40 gene expression in the presumptive medial NBs of row B. *vnd* may act through regulatory regions 5′ to *ac* to increase *ac* gene expression within one cell of the

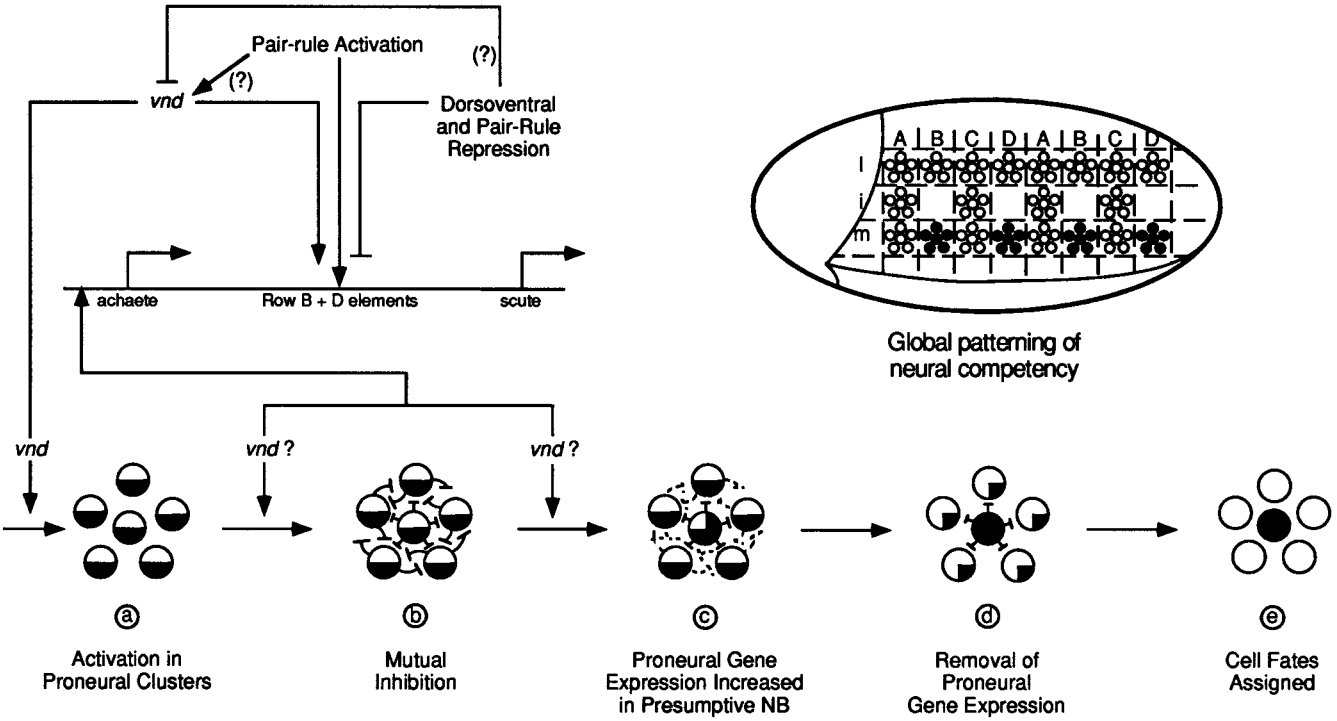

Fig. 5. The role of *vnd* in the patterning of and the assignment of cellular fates within proneural clusters. *vnd* is required for the formation of the medial proneural clusters of rows B and D (black circles within schematic of embryo). *vnd* activates proneural cluster formation in these regions through regulatory regions located 3′ to *ac* in either of two ways (see question marks, top of figure): it might act cooperatively with the gene products of the pair-rule and DV genes to activate proneural cluster formation; or the activities of the pair-rule and DV gene products might determine the pattern of *vnd* gene expression, which would in turn govern that of the proneural genes of the AS-C. Once, proneural gene expression has been activated within the medial cell clusters of rows B and D (a) *vnd* appears to act through regulatory regions 5′ to *ac* in order to help select which cell in these clusters becomes the NB. It is known that a competitive cell communication process (b; mutual inhibition) mediated by the neurogenic genes (e.g. *Notch* and *Delta*) helps single out the cell that becomes the neural precursor or NB (Heitzler and Simpson, 1991; Ruel et al., 1993). This process appears to result in one cell obtaining a higher level of proneural gene activity than the rest; this cell becomes the NB. It is possible that *vnd* acts to influence the process of mutual inhibition (arrow between a and b) by selectively increasing proneural gene expression in one cell within the cluster. The heightened level of proneural gene expression in this cell would allow it to compete with or inhibit more effectively the other cells in the cluster; thus, this cell would be favored to become the NB. Alternatively, the process of mutual inhibition may first single out one cell as the presumptive NB. This decision may then somehow trigger *vnd* to increase proneural gene expression within this cell (arrow between b and c). Once, one cell obtains a higher level of proneural gene activity (c), it triggers a process termed lateral inhibiton (mediated through the activity of the neurogenic genes) that inhibits and eventually removes the neural forming competency of the other cells in the cluster (d). Thus, the cell that retains proneural gene expression/activity becomes the NB (black) while all others are fated towards epidermal development (e).

proneural cluster and may therefore be involved in singling out this cell as the NB.

The function and regulation of the *vnd* gene

It appears that *vnd* acts at two levels during the process of NB formation and is required in a distinct subset of cells. With regard to proneural cluster formation, we can envision two ways in which *vnd* could influence *ac* gene expression through the 3′ regulatory region in the medial row B and D clusters (Fig. 5). First, *vnd* might be spatially regulated in a manner similar to *ac* and *sc* and selectively expressed in these clusters. Alternatively, *vnd* could be expressed in a broader domain but act as an obligate cofactor with the axis-patterning genes that activate proneural gene expression at specific anteroposterior positions within the medial column.

The expression of the B40 reporter gene in *vnd* mutant embryos suggests that *vnd* also functions later during NB segregation to help single out the cell that becomes the NB (Fig. 5), again selectively in the medial column of rows B and D. It is possible that like *ac*, *sc* and *l'sc*, *vnd* is expressed initially in cell clusters and then restricted to single cells. Retention of *vnd* expression in one cell would then increase proneural gene expression and favor this cell to become the NB. In this scenario, the dynamics of proneural gene expression would largely reflect those of the *vnd* gene. Clearly, the elucidation of the function, expression, and regulation of the *vnd* gene and its role in proneural cluster formation, NB segregation and NB identity (see below) awaits the cloning and characterization of the *vnd* gene product.

Regardless of the mechanism by which *vnd* activates proneural cluster formation and NB segregation, the fact that it selectively affects cells within the medial column of rows B and D raises the possibility that *vnd* is involved in specifying the medial identity of specific NBs. For example, in row B, the MP-2 and 3-5 SI NBs each arise from *ac/sc*-expressing clusters, MP-2 being the medial NB (Fig. 1). It is possible that *vnd* has a direct role in distinguishing the identity of MP-2 from 3-5 and other SI NBs. The selective role of *vnd* raises the

question of whether other, as yet unidentified genes act within rows A and C and/or within the lateral and intermediate columns to activate proneural cluster formation and NB formation and/or to specify NB identity. Since the loss of the AS-C and *vnd* removes roughly 50% of all NBs (Jimenez and Campos-Ortega, 1990), it is possible that other genes act to promote neurogenesis and to confer NB identity. Some of these could be zygotic genes, such as *vnd*, or there could be some maternal contributions to early neurogenesis. For example, the maternal contribution of the neurogenic gene *Notch* is sufficient to mask the zygotic role for *Notch* during the first, but not the subsequent waves of NB formation within the embryonic CNS (Struhl et al., 1993; see also Perrimon et al., 1986; Noll et al., 1993). The identification of other specific 'proneural genes', if they exist, should clarify issues concerning both the generation of the full complement of NBs and perhaps the determination of NB identity.

We thank Juan Modolell and Sonsoles Campuzano for providing AS-C cDNA clones and the B40 *ac-lacZ* reporter construct; and the late Carlos Cabrera for providing AS-C cDNA clones. We are grateful to Audrey Alberga for providing monoclonal antibodies generated against the Snail protein, before publication. We thank Chris Doe for his comments on the manuscript and permission to use his NB map as part of Fig. 1. We also thank Dervla Mellerick-Dressler for her critical review of the manuscript. We thank Kathy Vorwerk for expert technical assistance; Leanne Olds and Stephen Paddock for help with constructing the figures; and, Jamie Wilson for help with the preparation of the manuscript. This work was supported by a National Institutes of Health (NIH) predoctoral traineeship (GM-07215) and a Damon Runyon-Walter Winchell Cancer Research Fellowship (DRG 1279) to J. B. S., a NIH postdoctoral fellowship (GM-14441) to G. P., a National Science Foundation Presidential Young Investigators Award, the Shaw Scholar's Program of the Milwaukee Foundation, and the Howard Hughes Medical Institute.

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(Accepted 14 February 1994)