The role of the msh homeobox gene during Drosophila neurogenesis: implication for the dorsoventral specification of the neuroectoderm

Takako Isshiki¹, Masatoshi Takeichi¹,² and Akinao Nose¹,*

¹National Institute for Basic Biology, Myodaiji-cho, Okazaki 444, Japan
²Department of Biophysics, Faculty of Science, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-01, Japan

*Author for correspondence (e-mail: nose@nibb.ac.jp)

SUMMARY

Development of the Drosophila central nervous system begins with the delamination of neural and glial precursors, called neuroblasts, from the neuroectoderm. An early and important step in the generation of neural diversity is the specification of individual neuroblasts according to their position. In this study, we describe the genetic analysis of the msh gene which is likely to play a role in this process. The msh/Msx genes are one of the most highly conserved families of homeobox genes. During vertebrate spinal cord development, Msx genes (Msx1-3) are regionally expressed in the dorsal portion of the developing neuroectoderm. Similarly in Drosophila, msh is expressed in two longitudinal bands that correspond to the dorsal half of the neuroectoderm, and subsequently in many dorsal neuroblasts and their progeny. We showed that Drosophila msh loss-of-function mutations led to cell fate alterations of neuroblasts formed in the dorsal aspect of the neuroectoderm, including a possible dorsal-to-ventral fate switch. Conversely, ectopic expression of msh in the entire neuroectoderm severely disrupted the proper development of the midline and ventral neuroblasts. The results provide the first in vivo evidence for the role of the msh/Msx genes in neural development, and support the notion that they may perform phylogenetically conserved functions in the dorsoventral patterning of the neuroectoderm.

Key words: Drosophila, msh, homeobox, neurogenesis, CNS patterning

INTRODUCTION

Both in vertebrates and in invertebrates such as Drosophila, the central nervous system (CNS) arises from a two-dimensional structure, a homogeneous sheet of neuroectodermal cells. An early and important step in the generation of diverse sets of neurons and glia is the specification of neural precursor cells according to their position within the neuroectoderm along the anteroposterior (AP) and dorsoventral (DV) axes (reviewed by Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996; Doe, 1992; Broadus et al., 1995; Hartenstein et al., 1996; Liem et al., 1995; Shimeld et al., 1996). The spatial pattern of NB formation and previous cellular analysis in the grasshopper have suggested that a major factor in the generation of cellular diversity in the Drosophila CNS is the position at which a NB forms (reviewed by Goodman and Doe, 1993; Doe and Skeath, 1996). Recent studies showed that several msh/Msx genes of Drosophila are expressed in two longitudinal bands that correspond to the dorsal half of the neuroectoderm, and subsequently in many dorsal neuroblasts and their progeny. The acquisition of NB identity is the first and a crucial step in the generation of cellular diversity in the Drosophila CNS. This process is believed to be initiated before or at the time of NB delamination from the neuroectoderm. The stereotypy in the spatial pattern of NB formation and previous cellular analysis in the grasshopper have suggested that a major factor in the determination of NB identity is the position at which a NB forms (reviewed by Goodman and Doe, 1993; Doe and Skeath, 1996). The acquisition of NB identity is the first and a crucial step in the generation of cellular diversity in the Drosophila CNS. This process is believed to be initiated before or at the time of NB delamination from the neuroectoderm. The stereotypy in the spatial pattern of NB formation and previous cellular analysis in the grasshopper have suggested that a major factor in the determination of NB identity is the position at which a NB forms (reviewed by Goodman and Doe, 1993; Doe and Skeath, 1996). Recent studies showed that several segment polarity genes, including gooseberry-distal (gsb-d), wingless, hedgehog and patched, are regionally expressed in the neuroectoderm and specify the NB identities along the AP axis (Chu-LaGraff and Doe, 1993; Skeath et al., 1995; Bhat, 1996; Matsuzaki and Saigo, 1996). In contrast, the molecular mechanisms that specify NBs along the DV axis have been poorly understood.

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The msh/Msx genes are one of the most highly conserved families of homeobox genes (reviewed by Davidson, 1995). During vertebrate spinal cord development, Msx genes (Msx1-3) are regionally expressed in the dorsal portion of the developing neuroectoderm (e.g., Liem et al., 1995; Shimeld et al., 1996; Wang et al., 1996). Similarly in Drosophila, msh is initially expressed in two longitudinal bands that correspond to the dorsal half of the neuroectoderm, and subsequently in many dorsal NBs and their progeny (Lord et al., 1995; D’Alessio and Frasch, 1996; this study). These observations led to the suggestion that msh/Msx genes may play phylogenetically...
conserved roles in the DV patterning of the CNS (e.g., D’Alessio and Frasch, 1996; Wang et al., 1996). However, the function of this gene family during CNS development has remained largely unknown due to the lack of information on the mutant phenotype (see Discussion).

In this study, we report on the genetic analysis of msh in Drosophila neurogenesis. We show that msh loss-of-function mutations lead to cell fate alterations of a number of NBs formed in the dorsal aspects of the neuroectoderm, including a possible dorsal-to-ventral fate change. Conversely, ectopic expression of msh in the entire neuroectoderm severely disrupts the proper development of the midline and ventral NBs. These results strongly implicate this gene in the DV patterning of the neuroectoderm.

MATERIALS AND METHODS

Isolation and analysis of msh genomic DNA and cDNAs

Genomic DNA flanking the P-element insertion in the enhancer trap line rH96 was recovered by plasmid rescue. A 1.5 kb recovered sequence was then used to isolate overlapping genomic clones from a λFIXII genomic library (Stratagene). A DNA fragment adjacent to the P-element insertion point detected a transcript in a Northern blot analysis and was subsequently used to isolate several cDNAs from a 13- to 17-hour embryonic cDNA library (A.N., unpublished). Additional cDNA clones were also cloned by using the isolated cDNA as a probe. cDNA clones were sequenced by the T7 polymerase dideoxy chain method and autosequencer (ABI). The precise location of the P-element insertion in rH96 was determined by sequencing the genomic clone and rescued DNA.

Generation of msh alleles

The enhancer trap line rH96, which contained a P[ry‘, lacZ] insertion, was viable and expressed the normal level of msh transcripts. msh mutant alleles were generated by imprecise excision of the P-element in rH96. The transposon was mobilized by hybrid dysgenesis, and fertile and viable mutants or in sac-msh, msh68, egle-lacZ-lacZ /TM3 and msh68, hkb-lacZ /TM3 strains were generated by meiotic recombination between the transgenes and the msh allele. rH96 was recovered by plasmid rescue. A 1.5 kb recovered genomic clone and rescued DNA. Genomic DNA flanking the P-element insertion in the enhancer trap line rH96. The transposon was mobilized by hybrid dysgenesis, and fertile and viable mutants or in sac-msh, msh68, egle-lacZ-lacZ /TM3 and msh68, hkb-lacZ /TM3 strains were generated by meiotic recombination between the transgenes and the msh allele. rH96 was recovered by plasmid rescue. A 1.5 kb recovered genomic clone and rescued DNA. Genomic DNA flanking the P-element insertion in the enhancer trap line rH96. The transposon was mobilized by hybrid dysgenesis, and fertile and viable mutants or in sac-msh, msh68, egle-lacZ-lacZ /TM3 and msh68, hkb-lacZ /TM3 strains were generated by meiotic recombination between the transgenes and the msh allele. rH96 was recovered by plasmid rescue. A 1.5 kb recovered genomic clone and rescued DNA.

In situ hybridization to embryos

RNA in situ hybridization of whole-mount embryos was performed as described by Lehmann and Tautz (1994). Digoxigenin-labeled RNA probes were generated from a 0.6-kb fragment of msh cDNA cloned in pBluescript SK(+) (Promega), rabbit sAb against b-galactosidase (β-gal), mouse mAb against β-gal (Promega), rabbit sAb against EVE (Frasc et al., 1987), mouse mAb BP102 and 22C10 (Patel, 1994), mouse mAb against EN (Patel, 1994), rat sAb against RKB/REPO (Campbell et al., 1994), rat sAb against GCM (Jones et al., 1995), and rabbit sAb against TOLL (Hashimoto et al., 1991).

RESULTS

Identification of a P[lacZ] insertion in the msh gene

The enhancer-trap line rH96 was isolated and characterized for its expression in specific subsets of muscles and neurons (Klämbt et al., 1991; Nose et al., 1992). By standard methods (see Materials and Methods), approx. 10 kb of genomic DNA, spanning the P-element insertion site, was isolated (Fig. 1A). In this region, a single transcriptional unit of approximately 2.6 kb was detected by northern blot analysis of embryonic poly(A)+ RNA (data not shown), and the corresponding cDNA clones were subsequently isolated and sequenced. An open reading frame (ORF) that can encode a protein of 515 amino acid was found, a portion of which was identical to the partial sequence of the msh gene (Roberts et al., 1989). Thus, rH96 was a P-element insertion in the msh gene. While this work was in progress, two groups independently reported on the entire ORF of msh (Lord et al., 1995; D’Alessio and Frasch, 1996). The ORF deduced from our sequence data (submitted to GenBank accession no. AF09038) completely matches that reported by D’Alessio and Frasch (1996).

Expression of msh during neurogenesis

NB formation occurs between embryonic stage 8 to 11 in five phases (called S1-S5; Hartenstein and Campos-Ortega, 1984; Hartenstein et al., 1987; Doe, 1992). Early forming (S1-S3) NBs are mostly arranged in three longitudinal columns along the DV axis of the embryo. Later forming (S4 and S5) NBs are interspersed between the existing NBs. Along the AP axis of a segment, NBs are numbered from anterior to posterior as rows 1-7 (see Fig. 3). Recent in vivo tracing of DiI-labelled NBs showed that the positions of the NBs in the NB layer correlate with their site of origin in the neuroectoderm (Bosson et al., 1996). Accordingly, ventrally (medially) positioned NBs are derived from the ventral half of the neuroectoderm (thus referred to as ventral NBs); and dorsally (laterally) positioned NBs, from the dorsal half of the neuroectoderm (referred to as dorsal NBs). Due to the cell rearrangement within the neuroectoderm, the boundary between the ventral and dorsal NBs becomes a zigzag line at later stages (see Fig.

Immunohistochemistry

Antibody staining and dissection of embryos were carried out as previously described (Nose et al., 1992; Patel, 1994). The following primary antibodies were used: rabbit sAb against β-galactosidase (β-gal), mouse mAb against β-gal (Promega), rabbit sAb against EVE (Frasc et al., 1987), mouse mAb BP102 and 22C10 (Patel, 1994), mouse mAb against EN (Patel, 1994), rat sAb against RKB/REPO (Campbell et al., 1994), rat sAb against GCM (Jones et al., 1995), and rabbit sAb against TOLL (Hashimoto et al., 1991).
Fig. 1. Schematic representation of the msh gene and the lesions associated with mutant alleles. (A) Map of approx. 8 kb msh genomic DNA flanking the rH96 P[y' lacZ] insertion site. The location of the P-element insertion is indicated by the vertical line under the inverted triangle, with an arrow indicating the lacZ gene. The solid bar represents the SacI-PstI fragment used to isolate msh cDNAs. Hatched and open boxes below the map indicate the coding and 5' noncoding sequence of the msh gene, respectively. Restriction sites are abbreviated as follows: Ev, EcoRV; H, HindIII; N, NotI; S, SacI; X, XbaI. (B) Lesions associated with the four msh alleles generated by imprecise excisions of the P element. A break in the line indicates the deleted sequence with the broken line on each end representing the region including the break point. (GenBank accession no. AF009038).

3F). NBs, with a few exceptions, behave like stem cells, undergoing asymmetric cell divisions to bud off ganglion mother cells (GMCs), which divide once to generate two neurons or glia. Some NBs (e.g., longitudinal glioblast [LGB]) exclusively give rise to glia, and thus are actually glioblasts. However, for simplicity, they are also referred to as NBs throughout this paper.

Expression of msh during neurogenesis was studied by in situ hybridization for MSH RNA and immunohistochemistry for β-galactosidase (β-gal) in rH96. As described below, β-gal was expressed fundamentally in the same pattern as MSH RNA in rH96 (thus referred to as msh-LacZ). The neuroectodermal expression of msh was first detected at stage 5 as discontinuous patches in several segments, which later extended and merged to form bilateral stripes that ran along the length of the embryo (Fig. 2A; Lord et al., 1995; D’Alessio and Frasch, 1996). The msh-expressing domain corresponds approximately to the dorsal half of the neuroectoderm (Fig. 2B,C; D’Alessio and Frasch, 1996). From this region, four S1 NBs of the lateral column delaminate. Strong MSH RNA expression was only detected in one NB7-4 out of the four lateral NBs, although the other three NBs also expressed the transcript at a low level (Fig. 2B). In contrast, msh-LacZ was detected in all of the four lateral NBs (Fig. 2C). The initial msh expression in the dorsal neuroectoderm was transient and largely disappeared by late stage 9 (Fig. 2D).

Beginning at stage 10, msh expression was re-initiated in many dorsal S3-S5 NBs and their putative neuroectodermal proneural clusters (Fig. 2E-I, summarized in Fig. 3). These include the S3 NBs, NB6-4, LGB (longitudinal glioblast), and a putative glioblast (probably identical to the peripheral glioblast [PGB] described by Jones et al., 1995); S4 NBs, NB2-4, 4-3, and 5-4; and a S5 NB, NB3-4. MSH expression was

Fig. 2. msh is expressed in the dorsal neuroectoderm and NBs. Expression of MSH RNA (blue staining; A,B,D,E,G and H) and msh-LacZ (brown; C,F and I) in wild-type embryos during CNS development. In B,D,E,G and H, the embryos are additionally stained for EN (brown). In this and the following figures (Figs 3-7), anterior is up; the ventral midline is indicated by a vertical line. (A) Whole-mount embryo, lateral view. (B-I) dissected embryos, ventral view. Asterisks indicate the tracheal pits. (A) Low-magnification view of a late stage 8 embryo. (B) Similar stage to A but dissected. (C) msh-LacZ expression in four S1 NBs in the lateral column at stage 9. NB7-4s are indicated by white arrowheads. (D) MSH RNA expression in a late stage 9 embryo. Expression is only detected in NB7-4 (white arrowheads). (E) MSH RNA expression at stage 10 in newly formed S3 NBs, i.e., NB6-4 (white arrow), LGB (black arrow), and a putative peripheral glioblast (PGB). MSH is also detected in the proneural clusters of NB2-4 (gray arrow), NB4-3 (black arrowhead), and 5-4 (gray arrowhead). Inset shows the expression in LGB (left) and PGB (right). (F) msh-LacZ expression at stage 10. Expression in NB7-4 (white arrowheads), 6-4 (white arrow), and LGB (black arrow) is indicated. (G) MSH RNA expression at early stage 11. Expression is detected in newly formed S4 NBs, i.e., 2-4 (gray arrow), 4-3 (black arrowhead) and 5-4 (gray arrowhead) and in several other NBs that had initiated expression earlier. (H) MSH RNA expression at late stage 11. Expression in newly formed NB3-4 (thick arrows), GMCs of 4-3 (black arrowhead) and 5-4 (gray arrowhead), and in two final progeny of NB6-4, M-CBG and MM-CBG (white arrows), are indicated. (I) msh-LacZ expression at mid-to-late stage 11. msh-LacZ is detected in MSH-positive NBs and/or their progeny, including M-CBG and MM-CBG (white arrows), 2-4 (gray arrow), NB4-3 (black arrowhead), NB5-4 (gray arrowhead), and NB3-4 (thick arrow). The scale bar in A represents 50 μm, that in I represents 20 μm in B-I.
also seen in some of their immediate progeny (e.g., GMC; Fig. 2H). It is notable that all the msh-positive NBs are those derived from the dorsal half of the neurogenic ectoderm (Bossing et al., 1996), a region that had earlier expressed MSH (see Fig. 3F). In contrast, no MSH expression was seen in NBs derived from the ventral portion of the neuroectoderm. Some dorsal NBs (e.g., NBs 3-3, 3-5, 4-4, 5-5, 5-6), although they were derived from the msh-positive dorsal neuroectoderm, did not themselves express MSH.

During S3-S5 NB formation, msh-LacZ was expressed in the same NBs that expressed the RNA (Fig. 2F,I). Whereas MSH RNA expression in NBs was mostly transient and highest when they were initially formed, msh-LacZ persisted much longer and was expressed in the putative progeny of the msh-positive NBs. At stage 16, msh-LacZ was detected in many neurons and glia mostly located in the lateral portion of the nerve cord (see Fig. 4B). Some LacZ-positive cells were seen to be born in the lateral neuroectoderm and later migrate either medially towards the midline or laterally towards the periphery. From their characteristic migration pattern, morphology and their final locations, these cells were identified as glial cells, including six longitudinal glia, (LG1-6, derived from the LGB, see Fig. 4A,B), two cell body glia (MM-CBG and M-CBG, derived from NB6-4, see Fig. 2I), some channel glia (CGs, derived from NB7-4; G.M. Technau, personal communication), and some exit and peripheral glia (EGs and, PG2 and 3, T. Isshiki, M. Takeichi and A. Nose).
probably derived from the PGB; nomenclature of glia according to Klambt and Goodman, 1991; Ito et al., 1995).

**Generation of msh null mutations**

The msh gene had previously been mapped to the 99B region of the left arm of chromosome 3 (Roberts et al., 1989; Lord et al., 1995; D’Alessio and Frasch, 1996). No mutants that lacked msh function had been previously reported. The original P-element insertion in rH96, which was located at a position 171 bp upstream of the presumed transcription start site of msh (Fig. 1A), did not abolish the function of msh. We therefore generated four msh alleles, mshΔ60, mshΔ68, mshΔ138 and mshlacZΔ89, each of which contained a deletion in the msh gene by imprecise excision of the P-element (Fig. 1B, see Materials and Methods for details). All four alleles were embryonic lethal and failed to complement each other for lethality. Three of them, mshΔ60, mshΔ68 and mshΔ138, did not express detectable levels of MSH RNA, whereas mshlacZΔ89 expressed the transcript. mshΔ68 contained a deletion of approx. 4 kb that removed most of the first exon in which the translational initiation site and part of the ORF was localized. Thus this allele probably represents a null allele. mshΔ68 and mshlacZΔ89, exhibited indistinguishable CNS phenotypes (described below) either as homozygotes or as transheterozygotes. We thus assume that mshlacZΔ89 is also a null mutation and that the transcribed mRNA in this allele lacks msh function. In mshlacZΔ89, the lacZ gene in the P element was left intact and was normally expressed in the mshlacZΔ89/+ embryos. Thus the LacZ expression could be utilized to trace the cells that normally express msh in the mutant. We mainly used these two alleles, mshΔ68 and mshlacZΔ89, to analyze the mutant phenotype. The other two alleles, mshΔ60 and mshΔ138, displayed similar but slightly milder phenotypes than mshΔ68 and mshlacZΔ89 (data not shown).

**msh is required for proper development of dorsal NBs**

Since msh is expressed in the neuroectoderm before and during NB delamination, we first examined if NB formation took place normally in the msh mutants. Analysis of mshlacZΔ89 homozygous embryos showed that all the msh-LacZ-positive NBs formed normally (data not shown). Thus, msh is not required for the formation of NBs. We then examined the fate of two msh-expressing NBs, LGB and NB6–4. These NBs were chosen because all (for LGB) or part (for NB6–4) of their lineage had been previously characterized by specific markers (Jacobs et al., 1989; Ito et al., 1995; Higashijima et al., 1996). Furthermore, since many of their progeny normally migrate towards the midline away from other msh-LacZ-positive cells, their fate could be unequivocally followed by anti-β-gal staining in normal (rH96) and msh mutant (mshlacZΔ89) embryos.

The LGB is born in the lateral-most portion of the neurogenic ectoderm, where its first division is symmetrical. Then, the two progeny migrate medio-anteriorly, and interiorly towards the inner surface of the neuroepithelium, and divide further to generate six longitudinal glia, which later align along the longitudinal axon tracts. In normal (rH96) embryos, msh-LacZ was detected in the LGB and its progeny (Figs 2F, 4A, B). The development of the LGB lineage in msh embryos was analyzed by staining for msh-LacZ (in mshlacZΔ89 homozygous embryos), or for REPO, a glial-specific homeobox-containing protein (Xiong et al., 1994; Campbell et al., 1994; Halter et al., 1995). In msh embryos, LGB formed and conducted its first cell division, to produce two progeny, normally. However, their further cell division and migration were found to be abnormal. During early-to-mid stage 12, four LGB progeny that expressed msh-LacZ and REPO were seen in the medial region of the interior surface of the neuroepithelium in normal embryos (Fig. 4A, C). In msh embryos, 92% of the hemisegments (n=74) showed abnormality in the number and/or the position of the LGB progeny (Fig. 4D, F). Only two LGB progeny, which were larger than the normal LGB progeny at this stage, were present in many hemisegments, suggesting that they failed to conduct their second cell division to produce four cells. Many of them also failed to migrate properly and were found in the lateral and outer neuroepithelium close to the position where LGB had initially formed. Later, at stage 16, the msh-LacZ- or REPO-positive longitudinal glia were missing along the longitudinal axon tract (Fig. 4E).

The development of NB6–4 was studied by staining for msh-LacZ, REPO, and eagle-Kinesin-LacZ. The eagle-kinesin-lacZ fusion gene serves as a marker to visualize the morphology of eagle-positive NBs and their derivatives (Higashijima et al., 1996). NB6–4 is born in the lateral edge of the neuroectoderm, migrates medially, and divides in a characteristic quasi-symmetrical manner to generate two glial cells, MM-CBG and M-CBG (Fig. 5A). MM-CBG migrates further towards the midline and surrounds the VUM neurons by stage 13 (Fig. 5B). In msh embryos, NB6–4 formed normally and expressed specific markers such as REPO and eagle-Kinesin-LacZ. However, the timing and pattern of its subsequent cell division to generate MM-CBG and M-CBG appeared abnormal. Furthermore, their medial migration often failed to occur or was retarded (Fig. 5E). Their morphology, as visualized by eagle-Kinesin-LacZ, was also dramatically altered (Fig. 5F). The abnormality in the migration and/or the morphology of MM-CBG was observed in 71% of the hemisegments (n=39).

It should be noted that although patterns of cell division and migration were severely affected, both LGB and NB6–4 nonetheless expressed their specific markers (REPO for both LGB and NB6–4, and eagle-Kinesin-LacZ for NB6–4). GCM, a transcription factor that controls glia versus neuronal fate (Jones et al., 1995; Hosoya et al., 1995), was also normally expressed in the LGB and NB6–4 progeny in msh mutants (data not shown). These results suggest that msh is required for some aspects of LGB and NB6–4 development (e.g. cell division and migration) but not for others (e.g., specification as a glial cell).

Although lineage analysis was possible for only LGB and NB6–4, due to the lack of specific lineage markers for other NBs, we obtained evidence suggesting that the development of other dorsal NBs is also abnormal. For example, we observed abnormalities in the division and/or migration of several other glia, including some channel glia (progeny of NB7–4) and peripheral glia (putative progeny of PGB; Jones et al., 1995; data not shown). Abnormality was also seen in the eagle-Kinesin-LacZ-positive PM and PQ neurons (putative progeny of NB2–4 and/or 3–3; Higashijima et al., 1996: Fig. 5C, G). Presumably due to the improper migration and differentiation of many neurons and glia, the axon tracts of msh embryos were severely disrupted (Fig. 5D, H). The longitudinal connectives
were often reduced in width or completely missing between segments. The commissures were also distorted and somewhat fuzzy. However, development of ventral NBs appeared largely normal in the \textit{msh} mutant. EVE-positive GMC1-1a and aCC and pCC neurons (progeny of NB1-1), GMC4-2a and RP2 neuron (progeny of NB4-2), and CQ neurons (progeny of NB7-1) formed normally (see below). hkb-LacZ-positive medial cell clusters (putative progeny of NBs 1-1, 2-1, and 2-2; Chu-LaGraff et al., 1995) and identified neurons RP1 and RP3 (progeny of NB3-1) also formed normally (NB lineage according to Bossing et al., 1996).

**Fig. 4.** Aberrant migration and division of LGB in \textit{msh} mutant embryos. Dissected embryos, dorsal view. (A,D) \textit{msh}-LacZ expression in early stage 12 embryos. In the wild-type embryo (A), \textit{msh}-LacZ is detected in the four progeny of LGB (asterisks) in the inner surface of the neuroepithelium. In the \textit{msh} mutant embryo (D), the number and position of the LGB progeny are abnormal. The four LGB progeny are occasionally seen at the normal position (asterisks), but are often missing (upper-right and lower-left hemisegments). In the lower-right hemisegment, only two LGB progeny are seen (arrows). (B,E) \textit{msh}-LacZ expression in stage 16 embryos. \textit{msh}-LacZ-positive longitudinal glia are seen along the longitudinal axon tracts in the wild-type embryo (arrow, B), but are missing in the \textit{msh} mutant embryo (E). (C,F) REPO expression in early stage 12 embryo. In the wild-type embryo (C), four REPO-positive LGB progeny are seen in the inner surface of the neuroepithelium. In the \textit{msh} mutant embryo (F), only two REPO-positive LGB progeny were found at the normal position in many segments (black arrows). The LGB progeny, which failed to divide properly, are also seen at more lateral positions (white arrows; out of focus since they are located in a more exterior region of the neuroepithelium). The scale bar represents 20 \textmu m.

**Duplication of EVE-positive GMC4-2a and RP2 neurons in \textit{msh} mutants: a possible dorsal-to-ventral NB fate change**

A different type of \textit{msh} mutant phenotype was revealed by staining for EVE. EVE is normally expressed in a small subset of neurons including aCC, pCC, RP2, CQ, fpCC and EL neurons. In \textit{msh} mutants, an additional EVE-positive RP2-like cell was often detected adjacent to the normal RP2 neuron (56%, \(n=148\); Fig. 6A,B). At a lower frequency, approx. 2 additional EVE-positive cells were also seen adjacent to aCC/pCC neurons (see below). Except for the presence of these addi-

**Fig. 5.** Defects in the axon tract formation and the development of NB6-4, MM-CBG glia, and PM and PQ neurons in \textit{msh} mutant embryos. Dissected embryos, dorsal view. (A,E) Stage 12 embryos stained for \textit{eagle}-Kinesin-LacZ to visualize the NB6-4 progeny. In the wild-type embryo (A), NB6-4 has divided to produce two glial cells M-CBG (white arrow) and MM-CBG (black arrow), both of which have migrated medially. In the \textit{msh} embryo (E), the pattern of the NB6-4 cell division to produce the two progeny (white and black arrows) is abnormal. Furthermore, their medial migration failed to occur. (B,F) Same as A and E, but at stage 13. In the wild-type embryo (B), MM-CBGs (black arrow) with their characteristic elongated morphology are found at the midline. In the \textit{msh} embryo (F), MM-CBGs are often missing at the correct location at the midline (lower arrow) or show abnormal morphology (upper arrow). (C,G) Stage 13 embryos stained for \textit{eagle}-Kinesin-LacZ to visualize the PM and PQ neurons. In the wild-type embryo (C), PM (white arrowhead) and PQ (black arrowhead) neurons are found in the inner surface of the nerve cord. In the \textit{msh} embryo (G), these cells are often missing at their normal locations. Instead, cells with abnormal morphology (gray arrowheads) are seen in the inner surface at a more medial location. (D,H) Stage 16 wild-type (D) and \textit{msh} mutant (H) embryo labelled with MAb BP102 to visualize the axon tracts. The scale bar represents 20 \textmu m.
Fig. 6. Additional EVE-positive GMC4-2a and RP2-like cells in msh mutant embryos. Dissected embryos, dorsal view. (A,B) EVE expression in stage 16 wild-type (A) and msh (B) embryos. EVE is normally expressed in aCC/pCC (thick arrow), RP2 (black arrowheads), CQ (out of focus), and EL neurons (out of the field). In the msh embryo (B), additional RP2-like cells (black arrows) are observed adjacent to the normal RP2s (black arrowheads). (C) Early stage 12 msh lacZ-∆89 embryo double stained for EVE (brown) and msh-LacZ (blue), showing an ectopic EVE-positive GMC4-2a-like cell (large arrow) in the lateral region of the neuroepithelium. The GMC4-2a-like cell also expresses msh-LacZ, suggesting that it is derived from a msh-positive NB. Positions of the normal RP2 (large arrowhead) and RP2sib (small arrowhead) are also shown. They are located in a more interior region of the neuroepithelium, and thus are out of focus. (D) Same as C, but slightly later. A GMC4-2a-like cell (large arrow) is seen in a more medial position, closer to the normal RP2 and RP2 sib (large and small arrowheads). (E) Same as C and D, but at mid stage 12. By this stage, the GMC4-2-like cell has produced two EVE-positive progeny, an RP2-like (large arrows) and RP2sib-like (small arrows) cell. The inset and the boxed region in the main part of the figure show different focal planes of the same field. Note that the RP2-like cell has migrated interiorly so that it is now seen at the same focal plane as the normal RP2 (large arrowheads). (Diagram) EVE-positive NB4-2 and NB4-2-like lineage in wild-type and msh embryos. Orange, EVE expression; blue, msh-LacZ expression. The normal lineage of NB4-3 is not known. Note also the assignment of the origin of the additional EVE-positive cell to NB4-3 is tentative. The scale bar represents 10 μm.

Fig. 7. Ectopic expression of msh inhibits proper differentiation of the midline and ventral NBs but not that of dorsal NBs. (A) A whole mount embryo, ventral view; (B-K) Dissected embryos, dorsal view. (A) Stage 14 sca-msh embryo stained for BP102 showing severely disrupted axon tracts. (B,C) EN-positive cells in stage 13 wild-type (B) and sca-msh (C) embryos. EN-positive median NB progeny (arrows) are missing or greatly reduced in number in the sca-msh embryo. However, lateral EN neurons (arrowheads) formed normally. (D,E) hkb-LacZ-positive cells in stage 13 wild-type (D) and sca-msh (E) embryos. In the sca-msh embryo, hkb-LacZ-positive medial clusters (arrow) are missing. hkb-LacZ-positive lateral clusters (arrowheads) formed normally. (F,G) EVE-positive cells in early stage 12 wild-type (F) and sca-msh (G) embryos. EVE-positive aCC/pCC (large arrow in F) and RP2 (small arrow in F) neurons are almost completely missing in sca-msh (G). (H,I) EVE-positive cells in stage 16 wild-type (H) and sca-msh (I) embryos. In the sca-msh embryo, EVE-positive CQ neurons (arrow in H) are missing, whereas lateral EL cells (arrowheads) are present. (J,K) REPO-positive LGB progeny in early stage 12 wild-type (J) and sca-msh (K) embryos. LGB progeny formed normally in the sca-msh embryo. The scale bar in A represents 50 μm, that in K represents 20 μm in B-K.
tional EVE-positive cells, no abnormality was seen in the EVE expression pattern. The RP2 neuron is normally produced by a ventral NB, 4-2 (Doe, 1992). Since neither NB4-2 nor its progeny express msh, it is unlikely that the formation of the additional RP2 neurons was caused by some cell fate change within the NB4-2 lineage. An interesting possibility thus is that in the msh mutants, the fate of a msh-positive dorsal NB was transformed to a more ventral NB4-2-like fate, so as to produce the additional RP2-like cells. To test this hypothesis, we determined whether the ectopic RP2 arose from a msh-positive NB by double staining mshlacZ;Δ89 embryos for msh-LacZ and EVE.

During normal development of NB4-2, EVE is initially expressed in its first GMC, GMC4-2a, and then in its two progeny, a larger RP2 and smaller RP2sib (Patel et al., 1989; Doe, 1992). In mshlacZ;Δ89 embryos, at a stage when NB4-2 had already produced the normal EVE-positive RP2 and RP2sib, an additional EVE-positive GMC4-2a-like cell emerged in a position immediately internal to NB4-3 (Fig. 6C). This ectopic ‘GMC4-2a’ expressed msh-LacZ suggesting that it was derived from a msh-positive NB, most likely NB4-3. The later onset of EVE expression in the ‘GMC4-2’ compared with that in the normal GMC4-2 is also consistent with its having been derived from the later forming NB4-3. The ectopic ‘GMC4-2a’ produced two EVE-positive progeny, one larger and another smaller, just like the normal RP2 and RP2sib (Fig. 6D). The ‘RP2’ then migrated medially, changing their nuclear shape, in a characteristic manner similar to that of the normal RP2, and took a position adjacent to the normal RP2 by stage 13 (Fig. 6E). These results strongly suggest that in the absence of msh, the fate of NB4-3 was at least partially transformed to a NB4-2-like fate, so as to produce a NB4-2 lineage. Since the normal lineage of NB4-3 is unknown and no markers exist that can distinguish between NB4-2 and 4-3, it remains to be determined if the entire lineage of NB4-3 was converted to NB4-2 or the transformation occurred at the level of GMC. The analysis of the origin of the other ectopic EVE-positive cells found near aCC/pCC showed that they were also derived from a msh-LacZ positive NB(s) in the lateral CNS, suggesting that a similar dorsal-to-ventral cell fate transformation produced a duplicate ventral NB lineage (possibly NB1-1, the progenitor cell for aCC/pCC neurons) at the expense of dorsal NB(s) in msh mutants.

**Ectopic expression of msh severely inhibits proper development of the midline and ventral NBs**

To further analyze the function of msh during CNS development, we then studied the effect of ectopic expression of msh by using the GAL4-UAS system (Brand and Perrimon, 1993). We generated transgenic lines carrying UAS-msh transgenes and crossed them to the scabrous(sca)-GAL4 line that drives pan-neuronal expression (Klaes et al., 1994, see Materials and Methods for details). In the resultant sca-msh embryos, msh was expressed in the entire neuroectoderm and subsequently in all NBs.

Ectopic expression of msh resulted in a severe disruption of the axon tracts; the commissures were almost completely absent, and the longitudinal connectives were broken and interrupted (Fig. 7A). Furthermore, gaps were often seen along the midline, suggesting the malformation of the medial CNS. To analyze the effect of ectopic msh in more detail, we stained the sca-msh embryos for various specific markers. The results showed that development of the midline and ventral NBs was seriously perturbed. For example, TOLL-positive midline cells including the midline glia (Hashimoto et al., 1991; Nose et al., 1992) were often missing or showed abnormal morphology, although initial expression of TOLL in the midline precursor cells occurred normally (data not shown). Defects in the midline were also shown by the absence of En-positive median NB progeny (Patel et al., 1989, Doe, 1992; Fig. 7B,C). Many identified neurons located in the medial CNS also failed to differentiate in sca-msh embryos, including the EVE-positive aCC, pCC, CQ, fpCC, and RP2 neurons, all known to be derived from ventral NBs (Patel et al., 1989, Doe, 1992; Bossing et al., 1996; Fig. 7F-I), and hkb-LacZ-positive medial cell clusters (putative progeny of ventral NBs 1-1, 2-1, and 2-2; Doe, 1992; Chu LaGraff et al., 1995; Fig. 7D,E). EVE expression in GMCs 1-1a, 4-2a and 7-1a (Doe, 1992; Broadus et al., 1995) was also nearly absent, suggesting that ectopic msh affects early aspects of NB development (data not shown). The initial formation of ventral NBs, however, occurred normally (as revealed by SNAIL expression, data not shown). In contrast to the severe disruption of the midline and ventral NB lineage, development of neurons and glia derived from the dorsal NBs appeared to be largely normal. These include the EN-positive lateral neurons (Patel et al., 1989; Fig. 7C), EVE-positive EL neurons (Patel et al., 1989; Fig. 7J), and REPO-positive longitudinal glia (Fig. 7J,K).

**DISCUSSION**

**The role of msh during the development of dorsal NBs**

A prominent feature of msh expression is its restriction to the dorsal region of the developing CNS. It is expressed prior to NB delamination in longitudinal bands that correspond roughly to the dorsal half of the neuroectoderm. The initial msh expression in the dorsal neuroectoderm is transient and largely disappears by the time S2 NBs form, with the exception of the persistent expression in NB7-4. During later development, msh is re-expressed in a subset of S3-S5 NBs. Again, the expression is confined to those that delaminate from the dorsal neuroectoderm that had earlier expressed msh (see Fig. 3F).

The analysis of the msh loss-of-function mutants showed that msh is required for the proper development of dorsal NBs. During the lineages of LGB and NB6-4, the NBs form but fail to conduct their correct developmental program such as cell division and migration. Nonetheless, they express cell-type specific markers such as REPO, GCM, and eagle-Kinesin-LacZ, suggesting that part of their specification was executed normally. The defects thus appear not to reflect a simple cell fate transformation (e.g., to a more medial NB identity), but rather an inability to realize specific aspects of their normal fate. However, a phenotype suggestive of a dorsal-to-ventral fate change was observed for at least one dorsal NB. A msh-positive NB (most likely NB4-3) gives rise to EVE-positive progeny normally produced by a ventral NB, 4-2. These results suggest that msh can act as a switch between alternate fates along the DV axis for some NBs but not others.

In addition to the above examples, defects were seen in the...
requirement of aspects of NB development most likely reflect the intrinsic fate of NBs that will later delaminate from them. msh may also function autonomously in the NBs to specify their fate. It should also be noted that although the phenotypes seen in early aspects of NB development most likely reflect the intrinsic requirement of msh in the NBs and/or in the neuroectoderm, some of the defects seen during later development may also be partially due to the secondary effects caused by altered environment in the lateral neuroectoderm. For example, changed neighbor-relationship between GMCs and neurons due to the abnormalities in cell proliferation and migration may in turn induce changes in the fate of cells that will form later.

Another candidate gene for the DV specification of the neuroectoderm, ventral nerve cord defective (vnd), encodes an NK2-like homeodomain protein (Jiménez et al., 1995; Mellerick and Nirenberg, 1995). While msh is expressed in the dorsal neuroectoderm, vnd is transcribed in longitudinal bands that correspond to the ventral half of the neuroectoderm. Thus, expression of these two homeobox genes subdivides the neuroectoderm into two distinct domains along the DV axis. In vnd mutants, the expression of proneural genes in the medial neuroectoderm fails to occur; and, consequently, the formation of some medial NBs is disrupted (Skeath et al., 1994). The cell fate change of NBs in vnd mutants has not been studied. In contrast, in the msh mutant, NBs form normally but their subsequent development to generate specific lineage is affected. These observations suggest that these two genes may function in somewhat different manners during CNS development, although both are regionally expressed in the neuroectoderm.

**Disruption of ventral NB fate by msh**

Ectopic expression of msh in the entire neuroectoderm severely disrupted the differentiation of cells derived from the ventral neuroectoderm, indicating that restricted expression of msh is critical for proper CNS development. Although midline cells and ventral NBs initially formed, their subsequent development to generate specific GMCs and neurons was perturbed. The lack of EVE expression in GMCs1-1a, 4-2a and 7-1a, the first progeny of their parental NBs, indicates that early aspects of NB specification were affected. No obvious ventral-to-dorsal transformation was seen, however, suggesting that msh expression is not sufficient to convert ventral NBs to a dorsal fate. We observed that ventral markers such as vnd were still expressed largely normally in sco-msh embryos (T. I. and A. N., unpublished observations). Thus the defects seen in the ventral NBs appear to be caused by the concomitant execution of ventral and dorsal developmental programs. (Note, however, that we obtained preliminary results that the fate of some ventral NBs was partially transformed to a more dorsal one.) Due to the improper specification of ventral NBs (and the midline cells), and presumably also due to the changed environment that follows, most cells in the ventral neuroectoderm failed to differentiate or die, leading to a drastic disruption of the ventral CNS.

D’Alessio and Frasch (1996) recently reported that the msh expression domain in the neuroectoderm is expanded ventrally in the flb mutant. flb encodes a Drosophila EGF-receptor homolog (DER), and is required for the establishment of ventral fate in the neuroectoderm (Raz and Shilo, 1993; Golembi et al., 1996). This result suggests that msh is normally repressed in the medial neuroectoderm by the ventralizing signaling mediated by DER. Since ectopic expression of msh inhibits the proper differentiation of the ventral neuroectoderm, suppression of msh in this region during normal development must be an important component of DER function. Similarly, during vertebrate CNS development, the dorsally restricted expression of Msx1-3, and genes belonging to another family of homeobox genes, Pax3/7, is established in part by repressive action of a ventralizing signal, Sonic Hedgehog (SHH; reviewed by Tanabe and Jessell, 1996). Again, inhibitory events appear to play crucial roles in regulating vertebrate CNS patterning. Medial neural plate cells that are never exposed to SHH maintain Pax7 expression and lose their capacity to realize ventral fates (e.g., the floor plate and motor neurons; Ericson et al., 1996). Furthermore, ectopic expression of Pax3 inhibits floor plate differentiation (Tremblay et al., 1996). Thus, suppression of dorsally restricted transcription factors by ventralizing signals appears to be an essential element in DV patterning of the CNS both in Drosophila and in vertebrates.

**Possible roles of msh in DV patterning of the CNS**

Dorsally restricted expression of msh, its regulation by flb and other DV patterning genes (D’Alessio and Frasch, 1996), and the loss-of-function and gain-of-function mutant phenotypes described in this study, are all consistent with the notion that msh plays a role in specifying regional identity. msh may act as a transcriptional activator to confer dorsal positional identities to NBs. Alternatively, msh may function in a repressive manner to suppress ventral cell fate in the dorsal neuroectoderm. A role in transcriptional repression has been shown for murine Msx-1 (Zhang et al., 1996).

Since msh is expressed in the neuroectodermal region that corresponds to two to three longitudinal columns of dorsal NBs (see Fig. 3F), msh expression alone would not differentiate the DV identity of NBs within this domain. The lack of apparent dorsal-to-ventral cell fate transformation in LGB and NB6-4 in the loss-of-function mutants is also consistent with the idea that msh is only part of the mechanisms that generate the final NB specificity. We suggest that msh functions as a component of the genetic system that regulates NB identity along the DV axis.

The msh/Msx genes are an ancient family, whose members have been found in a variety of animal species (reviewed by Davidson, 1995). The high degree of sequence conservation in and around the homeobox, and the similar spatial expression pattern in vertebrate and Drosophila CNS suggest an interesting possibility that this gene family performs a conserved function in the DV patterning of the CNS. Two Msx mutations reported so far, a knock-out of the mouse Msx1 and a dominant mutation in the human MSX2, both display craniofacial abnormalities, with defects in bones and teeth (Jabs et al., 1993; Satokata et al., 1994). However, no gross abnormalities have been found in the neural tube, presumably due to redundancy among the Msx genes. The genetic analyses of msh presented in this study provide the first in vivo evidence
for the essential role of this gene family in neural development. Further studies on the function of msh in Drosophila should greatly help in elucidate the roles played by this gene family in CNS patterning.

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