The role of a Drosophila POU homeo domain gene in the specification of neural precursor cell identity in the developing embryonic central nervous system

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The Drosophila embryonic central nervous system (CNS) is derived from a stereotypic array of progenitor stem cells called neuroblasts (NBs). Each of the ~25 NBs per hemisegment undergoes repeated asymmetric divisions to produce, on average, 5–10 smaller ganglion mother cells (GMCs); each GMC, in turn, divides to produce two neurons. We demonstrate that the protein product encoded by a POU homeo domain gene (dPOU28/pdm-2) is expressed in the cell nuclei of a subset of NBs and GMCs. In the wild-type animal, GMC-1 is the only identified cell in the NB4-2 lineage that expresses dPOU28 protein to a high level, and it divides to produce the RP2 neuron and a second cell of unknown fate. Our results suggest that the presence of ectopically induced dPOU28/pdm-2 protein in the progeny of GMC-1 is sufficient to cause both of these cells to adopt their parental GMC-1 cell fate, leading to duplication of the RP2 neuron (and its sister cell) on the basis of both immunological and morphological criteria. These observations clearly implicate a role for dPOU28/pdm-2 in the specification of GMC-1 cell identity in the NB4-2 lineage and possibly in the specification of cell fate in other NB lineages in the developing embryonic CNS.

[Key Words: POU homeo domain gene; Drosophila; embryonic CNS; neural precursor cells]

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The formation of the Drosophila nervous system is dependent on a complex network of interacting developmental regulators. To rationalize the roles played by these regulators, the process of neurogenesis can be conceptually divided into two discrete categories of events: (1) the events leading to the formation of the neuronal progenitor cells, the neuroblasts [NBs] for the central nervous system (CNS) and the sensory mother cells [SMCs] for the peripheral nervous system (PNS) [for review, see Ghysen and Dambly-Chaudiere 1989; Campos-Ortega 1990; Jan and Jan 1991; Campuzano and Modolell 1992]; and (2) the events that specify the unique fate of each of the neuronal progenitor cells and each of their progeny [for review, see Doe 1992a].

A generally accepted perspective is that the anterior/posterior and dorsal/ventral pattern formation genes act to define a region of the ventral ectoderm, the neurogenic region, from where CNS precursors separate from the epidermal precursors [see Doe 1992]. Following gastrulation, through the combined actions of the family of proneural genes for which all of the characterized members, for example, daughterless and the achaete-scute complex, encode basic helix–loop–helix transcription factors [Villares and Cabrera 1987; Caudy et al. 1988], ~2000 ectodermal cells from the ventral neurogenic region acquire the potential to become neuronal precursors. However, only ~25% of these cells enlarge and delaminate from the ectoderm to form NBs; each of the forming NBs, through the actions of the family of neurogenic genes [e.g., Notch; for review, see Artavanis-Tsakonas and Simpson 1991], laterally inhibit their neuro-ectodermal neighbors and prevent them from also becoming NBs. In this way the combined actions of the proneural genes and the neurogenic genes act to ensure the formation of ~25 NBs per hemisegment. However, the available data suggest that the neurogenic and the proneural genes do not play a role in specifying the identity of neuronal progenitor cells [Jimenez and Campos-Ortega 1990; Rodrigues et al. 1990; Martin-Bermudo et al. 1991].

The NBs undergo a series of asymmetric divisions to produce, on average, 5–10 ganglion mother cells [GMCs], each of which divides to generate two neurons; studies on the grasshopper have led to the conclusion that each NB gives rise to a unique cell lineage, forming a characteristic family of neurons specific to that NB. The same is assumed for Drosophila because its nervous system is
highly homologous to that of the grasshopper (Thomas et al. 1984). However, in contrast to the grasshopper, NB lineages are not topologically distinct in Drosophila, and partial lineage information is available only for a few NBs [Doe et al. 1988a; Doe 1992a].

The identification of candidate genes that act to specify the unique identities of each of the NBs and their progeny GMCs and neurons has started only recently. Although a number of previously identified genes [Blochinger et al. 1990; Mlodzik et al. 1990; Dambly-Chaudiere et al. 1992], many of which act in pattern formation [e.g., Carroll and Scott 1985; Frasch et al. 1987; Gaul et al. 1987; Schroeder et al. 1988; Hooper and Scott 1989, Nakano et al. 1989; Patel et al. 1989a; van den Heuvel et al. 1989; Kania et al. 1990; for review, see Doe and Smouse 1990; Doe 1992a], appear to have a later phase of expression in the nervous system, a functional role in nervous system development has not been demonstrated for many of them. In addition, a number of genes have been identified that are necessary for the development of identified neurons in the CNS [Rothenberg et al. 1988; Thomas et al. 1988; Patel et al. 1989a; Finkelstein et al. 1990; Klambt et al. 1991]; however, their involvement in the specification of CNS cell fate is unclear. The two homeo box-containing pair–rule genes fushi tarazu (ftz) and even-skipped (eve) have loss-of-function phenotypes and expression patterns consistent with a role in the specification of GMC identity [Doe et al. 1988a,b]; the pair–rule gene runt is apparently required for the identity of the neuroblast, which gives rise to EL neurons [Duffy et al. 1991]; the phenotype of loss-of-function alleles of the homeo box gene prospero (pros) and its RNA expression pattern are consistent with it having a role in specifying NB identity [Doe et al. 1991]. However, pros protein has not yet been found in NBs [Vaessin et al. 1991]. At least two genes, cut [Bodmer et al. 1987; Blochinger et al. 1991] and pox neuro [Dambly-Chaudiere et al. 1992], function in the PNS in a way that is analogous to “NB identity genes”; these genes act to specify the identity of SMCs. Owing to the better defined lineage relationships for the PNS cell types, several other genes encoding putative transcription factors have been identified that specify cell fate at various levels of embryonic PNS development [for review, see Jan and Jan 1991].

To understand the generation of neuronal diversity in the CNS, the genes that act to specify neural precursor cell fate must first be defined. We and others [Billin et al. 1991, Dick et al. 1991, Lloyd and Sakonju 1991] have reported the isolation of the POU domain [Herr et al. 1988] genes, dPOU28/pdm-2 and dPOU19/pdm-1, whose transcripts accumulate in the developing CNS and PNS. Here, we assess the functional role of dPOU28/pdm-2 in CNS development. We present evidence for a specific and defined cell fate transformation in the developing embryonic CNS leading to the duplication of the RP2 neuron, which is caused by the ectopic expression of dPOU28/pdm-2. In addition, we have raised antibody that specifically recognizes the dPOU28/pdm-2 protein and show that this protein is expressed in a subset of NBs and GMCs [including GMC-1 of NB4-2]. Our results indicate that dPOU28/pdm-2 acts to specify the cell fate of GMC-1 in the NB4-2 lineage; its ectopic expression in the two progeny cells of GMC-1 is sufficient to cause both of these cells to adopt a GMC-1 cell identity. We discuss these results within the context of Drosophila CNS development and in light of biological functions defined for POU domain genes isolated from other organisms.

Results

Organization of dPOU28/pdm-2 and the generation of germ-line transformants carrying a HSP70/dPOU28 transgene

Previously, we and others have reported the sequence of two Drosophila genes, dPOU28/pdm-2 and dPOU19/pdm-1, with POU domains that are highly homologous to the mammalian Oct-1 and Oct-2 transcription factors [Billin et al. 1991; Dick et al. 1991; Lloyd and Sakonju 1991]. On the basis of their RNA expression patterns, we proposed a possible role for these genes in the determination of cell fate during the development of the embryonic CNS. Here, we test this assertion by examining the phenotypic consequences of ectopic expression of dPOU28/pdm-2. The genomic organization of dPOU28/pdm-2 is shown in Figure 1a. A construct in which the expression of a full-length dPOU28/pdm-2 cDNA was placed under the control of the HSP70 promoter was made [Fig. 1b; also see Materials and methods], and germ-line transformants homozygous for a single third chromosome insertion of the HSP70/dPOU28 transgene were generated [data not shown]. We show that dPOU28 protein accumulates ubiquitously in the cell nuclei of heat-shocked transgenic animals [Fig. 1c].

Ectopic expression of dPOU28/pdm-2 causes duplication of the RP2 neuron

Following heat shock during the early stages of embryonic CNS development, we looked for alterations in the pattern of previously identified neurons in the CNS using an array of antibody markers, including anti-en-grailed (en) [Patel et al. 1989b], anti-ftz (gift from the Gehring laboratory, Brozentrum, Basel, Switzerland), and anti-eve [Frasch et al. 1987]. We did not observe any obvious alterations in the anti-en and anti-ftz patterns [data not shown]. But with anti-eve we detected an alteration in the lineage of NB4-2, one of the few NBs for which a partial lineage [Fig. 2a] has been described [Doe et al., 1988b; Doe 1992a,b] and for which a number of antibody markers are available [see Fig. 2]. In wild-type animals, the neurons produced by the first two GMC progeny are marked. GMC-1 expresses both ftz and eve and divides to produce the RP2 neuron and a second cell of unknown fate; both RP2 and its sister cell express eve; the evidence for the GMC-1 lineage has been documented convincingly [Doe 1992b]. GMC-2 expresses ftz, and Doe [1992a] has suggested that it divides to produce the RP1 and RP3 neurons, both of which are marked by...
Duplication of RP2 is not caused by duplication of NB4-2

The two most likely explanations for the observed duplication of the RP2 neuron resulting from the ectopic expression of dPOU28/pdm-2 are: (1) NB4-2 is duplicated, or (2) some cell fate transformation has occurred within the NB4-2 lineage. The first possibility can be ruled out. If duplication of NB4-2 is the explanation, we would expect to see duplications of other identified neurons in the NB4-2 lineage (e.g., duplication of RP1 and RP3) in hemisegments where RP2 is duplicated. This possibility was tested by double-labeling heat-shocked and non-heat-shocked embryos with anti-eve (labels the RP2 nucleus) and mAb2D5, which recognizes fasciclin III (stains RP1 and RP3 cell bodies and axons; the RP2 cell body is stained at stage 12 but not in later stages; see legend to Fig. 4). In heat-shocked embryos, within those hemisegments in which two RP2 neurons are present, only one RP1 and one RP3 neuron are observed (Fig. 4b); however, the positions of the RP1 and RP3 neurons appear abnormal. In wild-type animals, RP3 is located directly ventral to RP1, whereas in the hemisegments in which RP2 is duplicated, RP1 and RP3 appear almost on the same plane of focus (see legend to Fig. 4). Nevertheless, these results argue against the duplication of NB4-2 as the cause for the observed RP2 duplication.

It is important to note that the conclusion against NB duplication is not dependent on knowing the GMC-2 lineage of NB4-2 because the temporal window during which ectopically expressed protein is required also precludes NB4-2 duplication as the cause of the RP2 duplication [see section on temporal considerations and Discussion, below]. Therefore, the most likely cause for the observed RP2 duplication is a change in cell fate within the GMC-1 lineage [see below].
Ectopic expression of \(d\text{POU}28/pdm-2\) causes the progeny cells of GMC-1 to adopt a GMC-1 fate within the NB4-2 lineage

In wild-type animals GMC-1 divides to produce RP2 and a sister cell of unknown fate [Doe 1992b]. There are at least two plausible rationalizations for the observed duplication of RP2 involving cell fate transformations within the GMC-1 lineage (also see Discussion): (1) The sister cell of RP2 now adopts the fate of RP2 and develops into a second eve-positive neuron, or (2) the two progeny of GMC-1 rather than developing into RP2 and its sister, now both adopt the fate of GMC-1. A comparison of the kinetics of appearance of eve-expressing cells from the NB4-2 lineage in wild-type [Fig. 5a–d] and heat-shocked animals [Fig. 5e–i] supports the latter possibility. In the wild-type NB4-2 lineage, the only identified GMC that expresses eve is GMC-1, and GMC-1 divides to generate two eve-positive cells, RP2 and its sister.

When the kinetics of appearance of eve-positive cells is followed in the wild-type NB4-2 lineage [1] there is initially one labeled cell [GMC-1]; [2] after GMC-1 divides, two labeled cells are observed; [3] eve expression fades from the RP2 sister cell [possibly because it is programmed to die] so that only one labeled cell [RP2] plus one weakly labeled cell [RP2 sister cell] are observed; and, finally, [4] only one labeled cell [RP2] is observed following the cessation of eve expression by the RP2 sister cell (possibly caused by cell death).

The kinetics of appearance of eve-labeled cells in heat-shocked animals (see Fig. 5e–i) is what would be expected if the progeny of GMC-1 adopt the fate of GMC-1: [1] Initially, there is one labeled cell [GMC-1]; [2] followed by the appearance of two labeled cells (the two converted GMC-1s); [3] four labeled cells are then observed [apparently because both GMC-1s divide to produce eve-positive daughters as in the wild-type situation]; [4] followed by two labeled cells and two weakly labeled cells [the labeled cells being RP2s, and the weakly labeled cells being RP2 sisters; finally, [5] two labeled cells are observed (the duplicated RP2s).

If the duplication of RP2 was the result of the sister of RP2 adopting the fate of RP2, at no time should four eve-positive cells be observed [near the RP2 location] following heat shock, because four labeled cells are observed, this possibility is unlikely. Moreover, the observed kinetics of appearance of eve-positive cells following heat shock clearly support the notion that the duplication of RP2 is the result of the generation of two cells with a GMC-1 identity. In addition, the physical proximity and the temporally synchronized appearance of the two eve-positive GMC-1s and their four eve-positive progeny following heat shock [see Fig. 5] strongly argue for the fact that the two GMC-1s are siblings; for example, if GMC-2 were to adopt a GMC-1 cell fate as a result of heat shock, one would expect the appearance of the two GMC-1s to be separated, temporally, by at least 30 min and one would not expect to observe the simultaneous appearance of two, followed by four, physically closely associated eve-positive cells as seen in Figure 5. These results and considerations lead us to conclude that the ectopic expression of \(d\text{POU}28/pdm-2\) causes both progeny of GMC-1 to adopt a GMC-1 cell fate (see Discussion).

Temporal considerations suggest that the observed cell fate transformations require the presence of ectopically expressed \(d\text{POU}28/pdm-2\) protein in the progeny cells of GMC-1

Our previous experiments have shown that a 10-min heat shock during stage 11 induced duplication of the RP2 neuron. We set out to determine more precisely the critical period during which heat shock is sufficient to effect the observed cell fate transformations. Because the
Figure 3. Ectopic expression of dPOU28/pdm-2 causes duplication of the RP2 neuron. (a–d) Anterior is at top; the midline is indicated by broken lines; thoracic segments are shown; RP2s are indicated by arrows; aCC and pCC are indicated by arrowheads. (a) Dorsal view of a non-heat-shocked stage 14 HSP70/dPOU28 embryo, dissected and stained with anti-eve. Note the presence of a single RP2 neuron (arrow) per hemisegment. (b) Same as a but double-labeled with anti-eve (black nuclear staining) and mAb22C10 (brown, cell surface and axon staining); note that the eve-positive RP2 neuron (arrow) appears to send its axon toward the posterior root of the ipsilateral ISN (thin arrow). (c) Dorsal view of a heat-shocked HSP70/dPOU28 embryo dissected and stained with anti-eve. Note the presence of two eve-positive cells per hemisegment at the RP2 position (arrows). (d) Same as c except double-labeled with anti-eve (black) and mAb22C10 (brown). Note that both of the eve-positive cells occupying the RP2 position appear to send axons toward the posterior root of the ipsilateral intersegmental nerve (ISN) (thin arrow). The eve-positive cells located near aCC (anterior arrowhead) and pCC (posterior arrowhead) are CQ neurons that normally lie ventral to aCC and pCC. Note that anti-eve stains three clusters of neurons, the aCC/pCC/CQ cluster, the EL cluster and RP2/RP2 sister cell (see Patel et al. 1989a); the position of RP2 and its progenitor are distinct from the other two clusters of stained cells. In this and all subsequent figures involving anti-eve staining, we focus only on RP2 and its relatives.

strains carrying the HSP70/dPOU28 transgene have a slower rate of development than that of wild type, the time of heat shock is given relative to the time of appearance of the eve-positive GMC-1 and the time of GMC-1 division (see legend to Fig. 6). Half-hour embryo collections from the HSP70/dPOU28 homozygous transformants were aged for various periods of time before being submitted to a 10-min heat shock at 37°C. The heat-shocked embryos were aged and scored for duplication of RP2. The results, schematically summarized in Figure 6, indicate that a 10 min heat shock during a 1-hr temporal window spanning the time of GMC-1 birth and GMC-1 division is sufficient to cause duplication of the RP2 neuron. However, heat-shocks before or after this temporal window do not cause RP-2 duplications at a significant frequency.

Two relevant pieces of information necessary for interpreting these results are the time lag between the time of heat shock and the appearance of (HSP70-driven) dPOU28/pdm-2 protein, as well as the stability of the ectopically expressed protein. Results of experiments in which embryos were exposed to a 10-min heat shock and then aged for various periods before being subjected to anti-dPOU28 staining indicate that accumulation of ectopically expressed dPOU28/pdm-2 protein reaches a maximal level 1 hr after heat-shock. High levels of protein accumulation persist until at least 2 hr after heat shock (data not shown). Extrapolating from these results, a 10-min heat shock during the critical 1-hr window will lead to the accumulation of high levels of dPOU28/pdm-2 protein in the nuclei of the progeny of GMC-1. These results are consistent with the notion that it is the presence of the ectopically expressed dPOU28/pdm-2 protein in the progeny of GMC-1 that causes these cells to adopt a GMC-1 cell fate.

GMC-1 is the only identified cell that expresses dPOU28/pdm-2 protein to a high level in the wild-type NB4-2 lineage

All of our previous results lead us to conclude that the presence of ectopically induced dPOU28/pdm-2 protein
in the progeny of GMC-1 is sufficient for these cells to adopt a GMC-1 cell fate. Implicit to this conclusion is that dPOU28/pdm-2 acts in the wild-type NB4-2 lineage to specify GMC-1 cell identity. These results and considerations predict that within the wild-type NB4-2 lineage, the dPOU28/pdm-2 protein should be expressed in GMC-1 but not its progeny.

To test this prediction, the dPOU28/pdm-2 protein expression pattern in the wild-type CNS was examined. Fusion protein containing a nonconserved region of the predicted dPOU28/pdm-2 protein [see Materials and methods] was used to generate polyclonal serum. The affinity-purified antibody specifically recognizes the dPOU28/pdm-2 protein as demonstrated by the observations that [1] the protein expression patterns parallel the RNA expression patterns [not shown], [2] there is a lack of staining in animals deficient for the dPOU28/pdm-2 gene [not shown], and [3] ubiquitous staining occurs in response to heat shock in transformants containing the HSP70/dPOU28 transgene [Fig. 1c]. The staining observed with anti-dPOU28 is nuclear, as would be expected for most proteins containing DNA-binding motifs. In wild-type animals the antibody labels a subset of NBs and GMCs [Fig. 7].

Within the early part of the wild-type NB4-2 lineage, the only cell that expresses the dPOU28/pdm-2 protein to a high level is GMC-1; the criteria for making this assignment are by position (the GMC-1 of NB4-2 that stains is almost always directly dorsal to NB4-2; see Fig. 7 and legend) and by double-labeling experiments demonstrating that the dPOU/pdm-2 positive GMC-1 also expresses eve [data not shown]. GMC-2, RP1, and RP3 do not express dPOU28/pdm-2 protein [data not shown]. We have observed weak transient staining in newly divided GMC-1 progeny, which is at a greatly reduced level compared with the high levels present in GMC-1; we believe this weak staining is the result of the persistence of the dPOU28/pdm-2 protein present in GMC-1 and not its de novo synthesis in the GMC-1 progeny. Similarly, we have also seen occasional dPOU28/pdm-2-positive NB4-2s. They are always cells that have just delaminated into the NB layer; older NB4-2s do not stain. Because dPOU28/pdm-2 is expressed in the neuroectoderm, we believe that the occasional staining observed is the result of carryover of the protein synthesized before segregation of NB4-2.

**Discussion**

A model for dPOU28/pdm-2 action

We have shown that the ectopic expression of dPOU28/
pdm-2 causes duplication of the RP2 neuron. The RP2 duplication is not caused by a duplication of NB4-2 because the RP1 and RP3 neurons, the putative progeny of GMC-2 in the NB4-2 lineage, are not duplicated; we stress that this conclusion is not dependent on knowing the GMC-2 lineage because the temporal window during which heat shock induces RP2 duplication also argues against duplication of NB4-2 as the cause of the RP2 duplication. By following the kinetics of appearance of eve-positive cells from the NB4-2 lineage in heat-shocked transformant embryos, we observe two cells that exhibit characteristics consistent with a GMC-1 identity; they express eve, they divide to form two RP2 neurons and two cells that transiently express eve (sister of RP2). The temporally synchronized appearance and close physical proximity of the two GMC-1s argue against [but do not preclude] the possibility that the second GMC-1 is caused by GMC-2 adopting the fate of GMC-1. The observed temporally synchronized appearance and physical proximity of the two GMC-1s suggest further that they are sibling cells. On the basis of these data, we conclude that as a result of dpOU28/pdm-2 ectopic expression, the two progeny of GMC-1 adopt the identity of GMC-1. The temporal window during which heat shock is required for the observed cell fate transformations suggests that the presence of ectopically expressed dpOU28/pdm-2 protein in the progeny of GMC-1 is sufficient to cause these cells to adopt a GMC-1 cell fate; consistent with this conclusion is the finding that in wild-type animals, the progeny of GMC-1 do not produce dpOU28/pdm-2 protein to an appreciable level. These results and considerations imply that the dpOU28/pdm-2 protein normally acts to specify GMC-1 identity in the NB4-2 lineage. This conclusion is supported by the finding that in the wild-type NB4-2 lineage, GMC-1 is the only cell identified that expresses dpOU/pdm-2 protein to a high level. A simple model that accounts for our results on the NB4-2 lineage following the ectopic expression of dpOU28/pdm-2 is represented schematically in Figure 8. Although the results are consistent with our preferred model (Fig. 8), there are several possible alternative explanations that cannot be eliminated. First, it is possible that the observed cell fate transformations are not cell autonomous events triggered by the ectopic expression of dpOU28/pdm-2 protein in the progeny cells of GMC-1; rather, the cell fate transformations caused by ectopic dpOU28/pdm-2 expression result from the disruption of normal cell–cell interaction events required for the development of the NB4-2 lineage. Although we cannot eliminate this possibility, results from both neuroblast ablation studies (Doe and Goodman 1985) and experiments using in vitro cultures of isolated Drosophila neuroblasts (Huff et al. 1989) indicate that the specification of the cell fate of the early GMCs is not affected by environmental influences or cell–cell interaction.
Drosophila POU gene specifies CNS precursor cell fate

Figure 6. The temporal requirement of a 10-min heat shock necessary to cause RP2 duplication. The timing of the early events in the development of NB4-2 lineage is shown schematically. Embryos homozygous for the HSP70/dPOU28 transgene were obtained using a 30-min collection and aged for various times at 25°C. Half of the embryos were fixed, stained with anti-eve, and examined. The rest of the sample was subjected to a 10-min heat shock at 37°C and aged to approximately stage 14, stained with anti-eve, and scored for RP2 duplication. Note that the growth rate of this stock at 25°C is ~80% of wild type, for example the presence of the eve-positive GMC-1 in wild-type animals occurs at ~6 hr, whereas its appearance in this stock occurs at ~7.5 hr. The temporal window during which a 10-min heat shock results in RP2 duplication in a large proportion of thoracic hemisegments is indicated by the solid bar. The data used are shown below the time line. (for details, see text).

POU factors and the regulation of DNA replication

The cell fate transformations induced by ectopically expressed dPOU28/pdm-2 protein cause two cells (the postmitotic RP2 and its sister cell), which no longer divide, to adopt the fate of a cell (GMC-1) that undergoes a further round of division. This clearly argues that dPOU28/pdm-2, presumably as a consequence of its role in specifying GMC-1 cell identity, plays at least an indirect role in regulating DNA replication and cell division.

From the perspective of DNA replication and cell division, our findings offer an interesting functional parallel with several mammalian POU genes.

POU genes and the development of blast cells

A number of mammalian (for review, see Rosenfeld 1991; Ruvkun and Finney 1991), Caenorhabditis (Finney...
Figure 7. dPOU28/pdm-2 protein is expressed in a subset of NBs and GMCs, including GMC-1 of NB4-2. [a, b] Ventral views of the same stage 11 embryos stained with anti-dPOU28 at two focal planes; midline is indicated by a broken line, anterior is toward the top. [a] The embryo is focused primarily on the plane of the NBs. NB4-2, which does not express dPOU28/pdm-2, is indicated by the large arrow; NB3-2 and NB5-3, which do express dPOU28/pdm-2, are indicated by small arrows [note the nuclear staining]. [b] The same hemisegment focused on a more dorsal plane to see the GMCs more clearly. The nucleus of the GMC-1 [arrowhead] of NB4-2 located immediately dorsal to NB4-2 is stained intensely; NB3-2, which can still be seen at this more dorsal focal plane, is indicated by the arrow. Many embryos have been observed, and the assignment of the GMC-1 of NB4-2 is unambiguous. Anti-dPOU28 antibody also stains a number of other NBs and GMCs.

and Ruvkun 1990), and Drosophila (Johnson and Hirsch 1990; Billin et al. 1991; Dick et al. 1991; Lloyd and Sakonju 1991; Treacy et al. 1991, 1992) POU domain genes show spatially restricted patterns of expression, with many being expressed in the nervous system. However, only for pit-1/GHF1 and unc-86 is there any information pertaining to genetic function. It is particularly interesting to note the similarities between the nematode unc-86 gene and dPOU28/pdm-2. In Caenorhabditis elegans, unc-86 is required in several NB lineages to differentiate the daughter cells from their mothers; in the absence of unc-86 function, the daughter cells take on the characteristics of their NB mothers. In several NB lineages, this gene is expressed asymmetrically in one of the two NB progeny. It is not known whether unc-86 is merely required or whether it plays an active role in specifying the fate of the NB progeny. Our data on the expression and function of dPOU28/pdm-2 in the NB4-2 lineage show several parallels with the unc-86 situation; the dPOU28/pdm-2 protein is expressed asymmetrically in one of the two NB progeny; it appears to play an active role in specifying GMC-1 identity, making it different from its daughters. Similarly Pit-1/GHF1 also appear to be required for the growth of pituitary blast cells. Therefore, all three POU genes [unc-86, pit-1/GHF1, and dPOU28/pdm-2], for which there exists information regarding in vivo function, appear to play a role in the development of blast cells.

The role of dPOU28/pdm-2 in the context of Drosophila CNS development

That the dPOU28/pdm-2 protein contains a putative DNA-binding POU domain and is located in the cell nucleus supports the notion that its function is effected through its action as a transcription regulator. It appears that the expression of important master transcription regulators in different spatial and temporal contexts is a common strategy adopted for the generation of cell diversity in a variety of developmental systems. Doe [1992a] has proposed that in the developing CNS, genes are expressed regionally in the neurogenic region that provide positional cues; in response to these cues, a set of NB identity genes are expressed in overlapping subsets of NBs. Within any given NB there is a unique combination of

Figure 8. A schematic representation of the altered NB4-2 lineage following ectopic expression of dPOU28/pdm-2. In wild-type animals GMC-1 expresses high levels of dPOU28/pdm-2 protein, but its progeny do not. However, if dPOU28/pdm-2 protein is ectopically induced in the GMC-1 progeny, they both adopt the fate of GMC-1. The consequence of these cell fate transformations on the NB4-2 lineage is depicted in the diagram (for details, see text).
these gene products and through their combinatorial action, a unique NB identity (and lineage) is determined. The identities of GMCs and neurons are specified by a set of GMC and neuronal identity genes, which are expressed in subsets of GMCs in response to the NB identity genes. The great majority of the candidate specification genes encode transcription factors; however, very few genes have actually been shown to be involved in specifying neural cell fate during CNS development (see introductory section). Within the context of this model, dPOU28/pdm-2 acts as a GMC identity gene, which specifies the identity of GMC-1 in the NB4-2 lineage. Because in addition to the NB4-2 lineage, dPOU28/pdm-2 protein is also expressed in ~13 identified NBs and a similar number of GMCs per hemisegment (S. Yeo et al., unpubl.), dPOU28/pdm-2 may play a more general role in the specification of cell fate involving other NB lineages.

The genes important for the formation of the NBs (the neurogenic and proneural genes) have largely been identified and analyzed extensively. However, understanding the mechanisms by which the diversity of CNS neuronal cell types is generated requires the identification of additional genetic elements involved in specifying the unique identities of each NB, their derivative secondary neuronal stem cells, the GMCs, and neurons. Like ftz and eve, dPOU28/pdm-2 protein is expressed in a subset of GMCs and acts to specify GMC identity. Interestingly, dPOU28/pdm-2 RNA (Lloyd and Sakonju 1991) and protein (S. Yeo, unpubl.) also show an earlier pairwise-rule-like pattern of expression at and shortly after cellular blastoderm; however, the functional significance of this expression pattern is unclear at present. Our results clearly implicate the POU homeo domain gene dPOU28/pdm-2 as a member of the growing family of genes that act to specify neural cell fate.

Materials and methods

Standard molecular biology techniques

DNA sequencing was performed by the chain-termination method with Sequenase (U.S. Biochemical). Southern hybridization and all routine DNA work was performed as described by Sambrook et al. (1989).

Antibody preparation

A fusion protein containing amino acid residues 19–212 from the nonconserved amino-terminal portion of the deduced dPOU28/pdm-2 protein was produced in Escherichia coli using a modified pGEX vector (Guan and Dixon 1991). The fusion protein was affinity purified using glutathione-Sepharose beads and injected into rabbits. Purified fusion protein (100 μl) was used for the initial inoculation (complete Freund's adjuvant) and subsequent boosts (incomplete Freund's adjuvant). Serum was affinity purified using the low pH method described by Sambrook et al. (1989).

Immunocytochemistry and photomicroscopy

Single-antibody staining was performed essentially as described by Yang et al. [1991]. Embryos were collected, dechorionated with 50% bleach, fixed with 4% paraformaldehyde for 30 min, devitellinized with a 1:1 mixture of heptane/methanol, washed with methanol, and rehydrated with PBT. Anti-eve and anti-ftz were rabbit polyclonal sera (gifts of Manfred Frasch (Mount Sinai Medical Center) and the Gehring laboratory, respectively); anti-fasciclin III (mAb2DS) was a gift of the Good- man laboratory (University of California, Berkeley), 22c10 was a gift from J. Campos-Ortega (University of Cologne, Germany). The mAb2DS stainings were performed using dissected embryos in the absence of detergent. Double-labeling was performed essentially as described by Kania et al. (1990). Following color development with the first antibody, the embryos were washed in 0.2 M glycine (pH 2.5) for 5 min. Embryos were washed with PBT and rebloacked with 1% BSA, and secondary antibody reactions were performed. Normally we use nickel-coupled DAB reaction for the first color development (black); the second color reaction was performed in the absence of nickel (brown). Photomicrographs were taken with a Zeiss Axiopt using DIC optics and either a 63× Planapo or a 20× Neofluor lens.

Heat shock construct

The BamHI site in the polylinker of the shuttle vector pHSX (gift from Rubin laboratory, referred to in Moses et al. 1989) was removed by cutting with BamHI, filling in the cohesive ends with Klenow, and religating the blunt ends. The Xhol–SalI fragment of the plasmid hsXS (gift from Susan Parkhurst, California Institute of Technology, Pasadena), containing the HSP70 promoter along with a downstream BamHI site, was cloned into the modified shuttle vector. The resultant plasmid was linearized with BamHI, and blunt ends were produced with Klenow; a HindIII–EcoRI fragment containing a full-length dPOU28/pdm-2 cDNA isolated from an embryonic cDNA library [Brown and Kafatis 1988] was blunt ended and inserted in the appropriate orientation into the filled-in BamHI site. From the resultant plasmid, the HSP70/dPOU28 gene was excised using NotI and cloned into the NotI site of the Drosophila germ-line transformation vector pCaWc (referred to in Moses et al. 1989).

Germ-line transformation

The procedure described by Spradling [1986] was used to obtain germ-line transformants. Preblastoderm rosy506 [ry506] embryos obtained from 45-min collections were dechorionated in 50% bleach, 0.01% Triton X-100, and arranged in rows of ~50 on a black nitrocellulose filter disc, with all of the posterior ends facing the same direction; the embryos were transferred to a coverslip made sticky using gum extracted from Scotch tape with their posterior ends facing the edge of the coverslip. The embryos were dissected for between 11 and 15 min by placing the coverslip in an air-tight plastic box filled with desiccant. After immersion in hydrocarbon oil (Volatex), the embryos were injected near their posterior end with a 500 μg/ml solution of the HSP70/dPOU28 construct in pCaWc. It was not necessary to coinject a plasmid to supply the P-element transposase because this construct already contains the P-element transposase gene in the portion of the molecule outside of the P-element inverted repeats. Injected embryos were allowed to develop at 22°C, and surviving adults were individually mated to ry506 flies. Progeny with ry+ eye color were obtained. Balanced stocks were established, and genomic blots were performed to ascertain the integrity, as well as the number, of inserts. Heat shocks were performed for 10 min at 37°C.
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Staging embryos

In general, we use the morphological criteria set out by Campos-Ortega and Hartenstein (1985). Figure 5 depicts a real sequence of events rather than a plausible sequence of events. There are two independent criteria that we use to stage embryos. The easiest one is the state of the anti-eve staining pattern. eve-positive cells first appear in the GMC-1 of NB4-2 and NB6-2 (late stage 10, early stage 11), followed by the GMC-1 of NB4-2 at mid-stage 11. During these early stages, staging can also be done with the NB arrays (Doe 1992b). The GMC-1 of NB4-2 can be unambiguously identified by position even in the absence of markers. The number of the eve-positive cells that give rise to the aCC/pCC/CQ cluster grows (up to about eight cells) during stages 11 and 12. The EL cluster of eve-positive cells first becomes evident as one or two cells during the middle of stage 12 and eventually increases to ~10 cells in the abdominal segments and ~6 cells in the thoracic segments. The size of the EL cluster reflects the relative age of the embryos from stage 12 onward.

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