Regulation of POU genes by *castor* and *hunchback* establishes layered compartments in the *Drosophila* CNS

Ravi Kambadur,^{1,4} Keita Koizumi,¹ Chad Stivers,¹ James Nagle,² Stephen J. Poole,³ and Ward F. Odenwald^{1,5}

¹The Neurogenetics Unit, Laboratory of Neurochemistry, and ²DNA Sequencing Facility, National Institute of Neurological Disorders and Stroke (NINDS), National Institutes of Health (NIH), Bethesda, Maryland 20892 USA; ³Department of Molecular Cellular and Developmental Biology, University of California at Santa Barbara, Santa Barbara, California 93106 USA

POU transcription factors participate in cell-identity decisions during nervous system development, yet little is known about the regulatory networks controlling their expression. We report all known *Drosophila* POU genes require *castor* (*cas*) for correct CNS expression. *drifter* and *I-POU* depend on *cas* for full expression, whereas *pdm-1* and *pdm-2* are negatively regulated. *cas* encodes a zinc finger protein that shares DNA-binding specificity with another *pdm* repressor: the gap segmentation gene regulator Hunchback (Hb). Our studies reveal that the embryonic CNS contains sequentially generated neuroblast sublineages that can be distinguished by their expression of either Hb, Pdm-1, or Cas. Hb and Cas may directly silence *pdm* expression in early and late developing sublineages, given that *pdm-1* cis-regulatory DNA contains ≥ 32 Hb/Cas-binding sites and its enhancer(s) are ectopically activated in *cas*⁻ neuroblasts. In addition, the targeted misexpression of Cas in all neuroblast lineages reduces Pdm-1 expression without altering Hb expression. By ensuring correct POU gene expression boundaries, *hb* and *cas* maintain temporal subdivisions in the cell-identity circuitry controlling CNS development.

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Nervous system development employs a cascade of integrated regulatory networks to generate specific cell lineages and, ultimately, create unique cellular phenotypes. A detailed understanding of these pathways remains a central goal of neurobiology. Cell-identity decisions during neural development are rooted in cell-cell communications that utilize both extrinsic and intrinsic cues to establish cell fate programs within stem cells called neuroblasts (NBs) (for review, see Artavanis-Tsakonas et al. 1995; Jan and Jan 1995; Bier 1997; Morrison et al. 1997). In the developing *Drosophila* CNS, different populations of NBs follow sequential ectoderm-delamination schedules, generating successive waves of NBs entering a sub-epidermal proliferative zone (Hartenstein and Campos-Ortega 1984; Bossing et al. 1996). Shortly after their arrival, most NBs initiate a series of 5–10 asymmetric divisions, producing a progenitor cell with each event—the ganglion mother cell (GMC). GMC divisions yield

either neurons or glia (for reviews, see Campos-Ortega 1993; Goodman and Doe 1993; Lin and Schagat 1997).

Although much is known about genes that participate in NB formation, that is, the proneural and neurogenic genes (for review, see Campos-Ortega 1993; Jan and Jan 1994), very little is understood about subsequent identity decisions, particularly those that establish differences between early and late developing NB sublineages. Underpinning the orchestrated arrival of NBs to their proliferative zone are cell fate decisions most likely controlled by temporally integrated transcriptional regulatory circuits. For example, the precise timing of neural identity gene expression is crucial for specific NB lineages (Yang et al. 1993; Bhat and Schedl 1994). In addition, the activation of certain sublineage identity genes has been linked to cell cycle or cytokinesis-dependent mechanisms (Cui and Doe 1995; Weigmann and Lehner 1995). Studies reported here reveal that during embryonic CNS development, NBs give rise to sequentially generated subpopulations of progeny distinguished by their selective expression of neural identity gene regulators.

⁴Present address: AgResearch, Hamilton, New Zealand.

⁵Corresponding author.

E-MAIL ward@codon.nih.gov; FAX (301) 496-1339.

Members of the POU homeobox gene family (Herr et al. 1988; for review, see Ryan and Rosenfeld 1997) play essential roles in establishing neuronal phenotypes in most, if not all, multicellular organisms, yet essentially nothing is known about the regulatory circuits controlling their dynamic CNS expression. The *Drosophila* genome contains four known POU genes: *pdm-1/dPOU-19/nubbin*, *pdm-2/dPOU-28/miti-mere*, *drifter/Cf1-a*, and *I-POU*, all of which are expressed in the developing CNS (Johnson and Hirsh 1990; Billin et al. 1991; Dick et al. 1991; Lloyd and Sakonju 1991; Treacy et al. 1991; Prakash et al. 1992; Bhat et al. 1994; Ng et al. 1995). In vivo functional analysis of three of the four *Drosophila* POU genes has shown that each is involved in cell-identity decisions during CNS development (for review, see Ryan and Rosenfeld 1997). The structurally related and coexpressed *pdm* genes are expressed in most, if not all, neuroectoderm cells during NB delaminations. Their expression during lineage development, however, is dynamic and maintained only in a subset of cells that make up each of the CNS ganglia (Billin et al. 1991; Dick et al. 1991; Bhat et al. 1994, 1995; Yeo et al. 1995). Unlike the identical expression patterns of the *pdm* genes, *drifter* (*drf*) and *I-POU* are activated relatively late in overlapping subsets of NB sublineages (Treacy et al. 1991; Anderson et al. 1995).

In previous reports, we and others have identified a sublineage CNS neuronal precursor gene, *cas* (also known as *ming*) (Cui and Doe 1992; Mellerick et al. 1992). Coding for a 792-amino-acid protein with a centrally located zinc finger domain, *cas* mRNA is expressed in many, if not all, late delaminating S3 through S5 NBs and in early S1–S2 NBs, but only after they have undergone several rounds of GMC-producing divisions (for NB delamination schedules, see Broadus et al. 1995; Bossing et al. 1996). Although *cas* may not be required for early NB sublineage development, its function is essential for many late developing sublineages, as evident from the reduced axon numbers observed in all CNS ganglia of late stage *cas*[−] embryos (Cui and Doe 1992; Mellerick et al. 1992).

To better understand the role of *cas* during CNS development, we have undertaken the biochemical analysis of its encoded protein, studied the dynamics of its NB lineage distribution, and have shown that *cas* function is required for the correct temporal expression of neural identity POU genes. DNA-binding studies have led to the unexpected finding that Cas shares DNA-binding specificity with another structurally different *Drosophila* zinc finger protein: Hunchback (Hb) (Tautz et al. 1987; Stanojevic et al. 1989; Treisman and Desplan 1989). During cellular blastoderm development, Hb functions as a repressor of *pdm* gene expression (Lloyd and Sakonju 1991; Cockerill et al. 1993). Studies reported here strongly support a role for both Hb and Cas as *pdm* repressors in early and in late CNS NB sublineages, respectively. We find that NBs sequentially produce subpopulations of progeny that can be identified by their expression of either Hb, Pdm, or Cas. Multiple Hb/Cas DNA recognition sites are part of the *pdm-1* cis-regula-

tory DNA, which also contains in vivo *cas* responsive enhancer(s), suggesting that Hb and Cas are direct transcriptional repressors of *pdm* expression. Finally, our studies show that *cas* function is required for proper expression of all known POU genes, and the effects of *cas* are both negative, repressing *pdm* expression, and positive, as *drf* and *I-POU* require *cas* for full expression. This differential control over neural identity genes suggests that Cas may have dual regulatory roles, functioning as an activator to ensure the expression of determinants that control cell fates in late forming NB sublineages and as a repressor to insulate their identity programs from factors that dictate earlier fates.

Results

Cas and Hb have similar DNA-binding preferences

Multiple features within the Cas primary structure suggest it may function as a DNA-binding transcription factor. It contains a centrally located zinc finger domain made up of four consecutive C₂-H₂C₂-H₂ repeats (Cui and Doe 1992; Mellerick et al. 1992). The second C₂-H₂ of each repeat closely resembles fingers of the *Xenopus* TFIIIA C₂-H₂ class (Miller et al. 1985). Flanking this repeat are motifs that may constitute either transcription transactivation or repression domains (Mellerick et al. 1992). In addition, UV-induced protein–DNA cross-linking in vivo studies revealed Cas binds genomic DNA (R. Kambadur and W.F. Odenwald, unpubl.).

To determine if Cas is a sequence-specific DNA-binding protein, we used the cyclic amplification of selected targets protocol (Wright et al. 1991). By use of a bacterially expressed Cas protein and anti-Cas antibodies, Cas–DNA complexes were immuno-selected from an unbiased set of degenerate dsDNA oligonucleotides (see Materials and Methods). After six rounds of selection/amplification, sequencing of cloned fragments revealed that all had at least one sequence motif in common and some contained two core recognition sequences (Fig. 1A). DNA fragments containing one site homologous to the consensus (Fig. 1C) produced a single prominent Cas–DNA gel shift and a fragment with two generated two complexes (Fig. 1A,B). Addition of Cas-specific antisera caused a supershift of the Cas–DNA complex (Fig. 1B); whereas addition of preimmune serum did not result in a supershift (data not shown). A randomly selected fragment that lacked the 10-bp core consensus failed to produce protein–DNA complexes with the recombinant Cas (Fig. 1B).

A search of known transcription factor DNA-binding sites showed that the Cas recognition sequence is almost identical to that of the *Drosophila* zinc finger protein Hb (Fig. 1C). The Cas consensus matches 9 bp out of 10 bp for the reported Hb sites (Stanojevic et al. 1989; Treisman and Desplan 1989). To determine if Cas binds Hb sites, we carried out gel-shift experiments by use of DNA fragments with exact sequence matches to Hb targets and found that Cas does bind to these sites (Fig. 7H, below; data not shown). The sequence-specificity of Cas–

tected in GMCs or in their progeny. Hb/Pdm-1 coexpression was also detected at similar frequency in early S1 and S2 NBs but not in their progeny. By stage 11, ventral cord Pdm-1-expressing cells are juxtaposed to the more dorsal or internal Hb-positive sublineages and flanked on their ventral/ventral-lateral side by the superficially positioned Cas-positive NBs and GMCs (Figs. 3 and 4). The same relative positioning of Hb, Pdm-1, and Cas subpopulations was also observed in the cephalic lobes, as Cas-expressing NBs and their offspring predominantly cover the outer flanks of Pdm-1 sublineages (Fig. 4D–F) whereas Hb-positive cells occupy deeper internal positions (data not shown). Although Hb and Cas immunopositive cells together make up >50% of the cells pre-

sent in stage 12 ganglia, no Hb/Cas coexpressing cells were detected in NBs or in their progeny (Fig. 3J,K). In fact, no cell at any stage of embryonic development was observed coexpressing these zinc finger proteins. Simultaneous labeling of Hb, Pdm-1, and Cas reveals that most, if not all, NB lineages express at least one of these transcription factors (Fig. 3K; data not shown). The absence of prolonged overlap between Hb/Pdm-1 coexpression or Pdm-1/Cas coexpression in early and late sublineages, respectively, suggests that Hb and Cas may control, via repression, the temporal boundaries of *pdm* expression during CNS development.

In situ mRNA localizations show that *cas* expression is predominantly restricted to CNS NBs, with little or no

Figure 2. (See following page for legend.)

message detected in GMCs and no detectable mRNA observed in newly formed neurons or glia (Mellerick et al. 1992). The embryo immunostaining data presented here, however, reveal that Cas protein persists significantly longer than its message and is found in the nuclei of many cells generated during late sublineage development (for message/protein dynamics, cf. Fig. 2 with Figs. 4 and 5 of Mellerick et al. 1992). Cas-positive nuclei in stage 14 and older embryos are detected in all CNS ganglia (Fig. 2H) and many most likely belong to nascent neurons. No immunostaining was observed in *cas* null embryos, which confirms that our anti-Cas polyclonal antibodies are specific for Cas and do not bind to structurally related proteins, (Fig. 9B, below; and data not shown). Like Cas, Hb, and Pdm-1 are also detected in all ganglia of stage 14 and older embryos (data not shown), suggesting that the regulatory functions of all three transcription factors may be required in many neurons and glia until their functional phenotypes have been achieved.

Hb and Cas are repressors of Pdm CNS expression

Given the apparent transient overlap between Hb and Pdm-1 expression in the CNS and Hb's established role as a repressor of *pdm* expression in the cellular blastoderm, it is likely that Hb also silences *pdm* expression during early NB sublineage development. Embryos lacking Hb function suffer multiple defects (for review, see Lindsley and Zimm 1992). During CNS development, *hb*⁻ embryos fail to develop labial and thoracic ganglia

and gaps form between the subesophageal maxillary neuromeres and the abdominal ganglia. In addition, the seventh and eighth abdominal segments are fused because of the absence of parasegment 13 (White and Lehmann 1986). To further characterize the phenotypic consequences triggered by the loss of Hb and compare them with defects caused by loss of Cas, we examined the axon fascicle organization in *hb* null embryos. As expected, immunostains with the BP102 Mab axon marker revealed that *hb*⁻ embryos have severe axon guidance defects. Missing are the highly ordered ventral cord axon scaffolds made up of longitudinal connective and commissural fascicles (Fig. 5A,B). Although axon fascicles do not form properly, however, the intensity of BP102 immunoreactivity in *hb*⁻ ganglia was similar to that observed in wild-type embryos, indicating that significant numbers of neurons still generate axons, albeit misguided, in *hb*⁻ embryos (Fig. 5A,B). Because many axon-guiding glia and pathfinding neurons are born from early NB sublineages, *hb* function may be essential for establishing correct axon guidance cues in these sublineages. Unlike the *hb*⁻ phenotype and consistent with its late NB expression, loss of *cas* does not disrupt the formation of axon connective or commissure fascicles, but it does reduce the number of late forming axons that participate in these fascicles (Cui and Doe 1992; Mellerick et al. 1992).

Pdm-1 immunolocalizations performed on *hb*⁻ embryos confirmed the hypothesis that Hb also functions as a repressor during CNS development. Comparisons between *hb*⁻ and wild-type Pdm-1 immunostaining pat-

Figure 2. Cas expression during embryonic CNS development. Dissected fillets or transverse sections (6 μ m thick) of whole-mount immunostained embryos reveal Cas expression dynamics during embryonic CNS development (embryo staging according to Hartenstein and Campos-Ortega 1984; anterior up in A–E, G, and H; ventral up in F). The same magnification was used in A–E; scale bar in E represents 80 μ m. In F, panels 1–5, the same scale bar represents 25 μ m, in G it equals 20 μ m, and in H it is equivalent to 35 μ m. (A) Stage 9 embryos stained with either Cas (main panel) or Hb (*inset*) antisera. Cas protein is detected first in segmental clusters of ventral midline mesectodermal cells (arrow) and in cells that line the anterior midgut primordium (arrowhead). At this time, no Cas expression is detected in either cephalic lobe or ventral cord NBs (see also panel F, J). The initial reduced expression observed in the thoracic and first abdominal midline cells reflects the delayed onset of *cas* mRNA expression in these segments (data not shown). (*Inset*) By late stage 9, NB delaminations from the lateral ventral cord neurogenic regions have produced three rows of Hb-positive NBs flanking the midline in all gnathal, thoracic, and abdominal segments (see also Fig. 3). Note, NBs were identified on the basis of their large cell body diameters and on their position underlining the ectoderm. (B) During stage 10, bilaterally symmetrical subsets of cephalic lobe and ventral cord NBs initiate Cas expression. The first ventral cord NBs to express Cas are the late delaminating NB6-1s located on the posterior edge of the gnathal, thoracic, and abdominal a1–8 segments (also see F, panel 2). At this time, some of the NB6-1s have produced Cas-positive GMCs (arrows). (*Inset*) Confocal colocalization views of Hb (green) and Cas (red) reveal no coexpression in NB6-1s or in midline precursors. (C) By early stage 11, the number of cephalic lobe Cas-positive NBs has nearly doubled and the number of Cas-expressing ventral cord NBs has increased to 5–7 per hemisegment (see also F, panel 3). (*Inset*) Following activation of Cas expression in NB6-1s, the next NBs to express Cas are the earlier delaminating (S1) NB5-2s (arrow). At this time, NB5-2s have terminated Hb expression and their Hb positive sublineages take up dorsal positions relative to ventrally located Cas-positive NBs (see Fig. 3). (D) During late stage 11, Cas expressing NBs in ventral cord neuromeres have now increased to 9–10 and 7–8 per thoracic and abdominal hemisegments, respectively. (E) Stage 12, germ-band contraction; by late stage 11/early stage 12, most CNS neuroblasts express Cas. Cas-expressing NB sublineages are positioned on the outer surface of the cephalic lobes and on the ventral/ventral-lateral surfaces of the developing subesophageal ganglion (arrow) and ventral cord neuromeres (see also F, panels 4 and 5; and Fig. 4). Also note, no Cas expression is detected outside the CNS. (F) Transverse sections (6 μ m thick) through abdominal segments of stage 9–13 embryos (1–5, respectively) reveal that Cas-expressing NBs, and their sublineages take up ventral/ventral-lateral positions in the developing neuromeres. (G) Cas is concentrated in GMCs during NB cytokinesis. Shown is a ventral view of a stage 11 ventral cord showing Cas-positive NBs and GMCs in the second and third thoracic and first three abdominal segments. Note, the more intense immunostained GMCs (arrows) relative to their weaker stained NBs. Also note, nuclear-localized Cas in nondividing NBs (arrowhead). In situ mRNA localizations show that *cas* is not expressed in GMCs (data not shown; Mellerick et al. 1992). (H) By stage 15, Cas immunopositive sublineages are evenly distributed on the outer cephalic lobe surfaces and on the ventral/ventral-lateral regions of the subesophageal and ventral cord ganglia.

terns showed that in the absence of Hb, Pdm-1 is ectopically expressed in all CNS ganglia (Fig. 5C and D). Transverse sections of Pdm-1 immunostained *hb*⁻ embryos also revealed that early NBs that normally do not maintain Pdm-1 expression after their ectoderm delaminations fail to terminate Pdm-1 expression (Fig. 5E). The presence of ectopic Pdm-1 is also found in many early GMCs and by stage 12, Pdm-1-positive cells occupy both dorsal and inner regions of the developing ventral cord neuromeres (Fig. 5F). The expanded dorsal-ventral zone of Pdm-1 expression in *hb*⁻ ventral cords indicates that many early GMCs and their progeny, normally marked by Hb expression, now ectopically maintain Pdm-1. Ectopic Pdm-1 expression, however, is not detected throughout the developing ganglia. After stage 11 in *hb*⁻ embryos there is no Pdm-1 expression in the most ventral regions of the ventral cord ganglia (Fig. 5F). Similarly, no ectopic Pdm-1 was detected along the outer/superficial surfaces of *hb*⁻ cephalic lobes after stage 11

(data not shown). This suggests that other mechanism(s) are regulating *pdm* expression in late developing sublineages.

Interestingly, loss of Hb did not markedly perturb Cas expression (Fig. 5H). Although activation of Cas in many NBs may be slightly delayed, the overall dynamics of its expression and order of NB activation was similar to that observed in wild-type cephalic lobe and abdominal ganglia. As in wild-type embryos, activation of Cas in *hb*⁻ embryos is first observed in ventral cord midline cells followed by the NB6-1s and then in NB5-2s (data not shown). In addition, the overall number of late developing sublineages in cephalic lobes and in abdominal ganglia of *hb*⁻ embryos appeared to be equivalent of that of wild-type embryos, as judged from Cas immunostaining in late stage 12 embryos (Fig. 5H).

To determine if Cas is also a *pdm* repressor, we next examined Pdm-1 and Pdm-2 expression in *cas* null embryos (Mellerick et al. 1992). In stage 9 and in younger

Figure 3. CNS neuroblasts produce layered sublineages distinguished by their expression of Hb, Pdm, or Cas. The dynamics of Hb (A,C,E,G) and Pdm-1 (B,D,F,H) expression during ventral cord development are shown in transverse (6 μ m thick) sections through abdominal segments of stage 9 (A,B), stage 10 (C,D), stage 11 (E,F), and stage 12 (G,H) embryos immunostained in whole-mount with either Hb or Pdm-1 antibodies. (A,B) Sections through stage 9 embryos reveal that most, if not all, fully delaminated NBs express Hb (see also Fig. 2A, inset) whereas Pdm-1 expression is restricted to neuroectoderm cells. (C,D) By stage 10, many NBs and GMCs are immunostained with Hb antibodies, however, not all NBs contain detectable levels of Hb (arrow). In addition to its high levels of neuroectoderm expression, low levels of Pdm-1 immunostaining are now detected in a small subset of NBs and GMCs. (E,F) Starting at late stage 10, and through stage 11, there is a progressive reduction in the number of NBs containing detectable levels of Hb, whereas many GMCs and/or their progeny maintain high levels of Hb immunostaining. Loss of Hb expression in NBs parallels the activation of Pdm-1 NB expression. The number of GMCs containing detectable levels of Pdm-1 also rises during stage 11 and follows that observed in NBs. Given their close apposition to Pdm-1-positive NBs, many of these GMCs were most likely products of Pdm-1-expressing NBs. (G,H) Stage 12; during this period, Pdm-1 NB expression is reduced such that only low levels of Pdm-1 immunostaining are found in NBs. Hb and Pdm-1-positive sublineages are now positioned dorsal to late dividing NBs. Scale bar in H is equivalent to 28 μ m and also applies to A–G. (I) By stage 13, Hb and Pdm-1 immunopositive sublineages form layered subpopulations of cells in all CNS neuromeres. In the ventral cord, Pdm-1 positive sublineages are juxtaposed to the more dorsal/internal Hb sublineages. Shown are lateral, confocal fluorescent views from a double-labeled, Hb (green) and Pdm-1 (red), stage 13 embryo (anterior is left and ventral up). (J) Confocal immunofluorescent views of stage 12 and older embryos reveal that Hb (green) and Cas (red) positive sublineages do not overlap and occupy the dorsal and ventral neuromere surfaces, respectively. Shown is a stage 13 ventral cord (lateral view; anterior is left and ventral up). Note the more ventral position of the Cas-expressing sublineages compared with the internal Pdm-1 positive cells in I. (K) Triple-labeling [Hb (blue), Pdm-1 (green), and Cas (red)] reveals that by stage 13 most, if not all, sublineages express one of these transcription factors. For confocal ventral views of adjacent Pdm-1- and Cas-expressing sublineages, see Fig. 4. Note, the arrow in K indicates a NB that coexpresses Pdm-1 and Cas. Scale bar in J is 30 μ m and also applies to I and K.

Figure 4. By mid-CNS development, Cas and Pdm-1 colocalizations reveal little overlap in their expression. Confocal immunofluorescent views of embryos doubled-labeled for Cas (red) and Pdm-1 (green) reveal that the activation of Cas expression in CNS NBs coincides with the loss of Pdm-1 NB expression. (A–C) Shown are ventral to dorsal (left to right) optical section views of a late stage 12 ventral cord (anterior, up). Note the more ventral/ventral-lateral position of the Cas-positive cells relative to the deeper/internal Pdm-1 immunostained sublineages. (D–F) Cephalic lobe expression of Cas and Pdm-1; shown are dorsal to ventral (left to right), serial views of a late stage 12 brain. Arrow in *E* points to a NB immunostained for both Cas and Pdm-1. Note, the red staining between and below the cephalic lobes is caused by the nonspecific binding of the rhodamine-tagged secondary antibody to yolk cells. Same magnification in all panels. Scale bar in *A*, 50 μ m.

embryos, no differences were detected between the *cas*[−] and wild-type expression patterns of Pdm-1 or -2. Starting at stage 10, however, we observed that NBs failed to terminate expression of both Pdm (Fig. 6). Ectopic Pdm expression was observed in most, if not all, late developing sublineages in all CNS ganglia. The sustained Pdm expression is most likely caused by transcriptional derepression, as *pdm-1* mRNA in situ hybridizations also reveal that its message persists in *cas*[−] late NBs (data not shown). In addition, *cas* function is required to silence *pdm-1* enhancer(s) (see below), whereas loss of *cas* function had no detectable effect on Hb expression (data not shown).

cas controls *pdm-1* by deactivating its neuroblast enhancer(s)

In vivo analysis of *pdm-1* genomic DNA has identified the main *cis*-regulatory elements controlling its embryonic expression (Fig. 7; K. Cockerill and S. Poole, unpubl.). These control elements lie within a 6.3-kb DNA fragment flanking the 5' side of its transcribed sequence.

Analysis of transformant embryos containing a P[*mini-white.pdm-1.GAL4*] vector (Fig. 7G) showed that the *pdm-1* regulatory DNA contains enhancer(s) that can drive the expression of both the *mini-white* and *GAL4* reporter genes in the same cells in which *pdm-1* is normally expressed (Fig. 7A,B). As with the endogenous *pdm* genes, in a *cas*[−] background both of the divergently transcribed transgenes are ectopically expressed in NBs during late sublineage development. This result shows that the enhancer(s) within the 6.3-kb regulatory DNA are negatively regulated by Cas (Fig. 7C–F).

To explore the possibility that Cas may play a direct role in silencing *pdm* gene expression, we carried out Cas-DNA immunoprecipitation with *pdm-1* promoter fragments to test for potential Cas DNA-binding sites (data not shown). DNA sequence analysis of bound fragments revealed 32 potential DNA-binding sites, all sharing at least 8 of the 10 bp with the Hb consensus sites (Fig. 7G). Gel-shift analysis and competition assays with *pdm-1* fragments containing exact matches to the Hb consensus sequences confirmed that Cas binds to these sites (Fig. 7H). In addition, base-pair substitutions in one of the sites showed that the A/T-rich core sequence is essential for Cas binding (Fig. 7H). All together, the results suggest that Hb and Cas regulate *pdm* expression by interacting directly with their *cis* regulators to deactivate controlling enhancer(s), with Hb repressing the *pdm* genes early and Cas silencing late in CNS development.

To test if Cas can silence Pdm-1 expression outside of its endogenous sublineage boundaries, we studied the effects of misexpressing Cas earlier in CNS development. By use of the *GAL4-UAS* targeted misexpression strategy (Brand and Perrimon 1993), we compared Pdm-1 expression in P[*prospero.GAL4*]/P[UAS.*cas*] embryos with that of wild-type controls. *prospero* is expressed in all NBs shortly after they delaminate (Doe et al. 1991; Vaessin et al. 1991); thus Gal4, expressed from the *prospero.GAL4* transgene, activates *UAS.cas* expression in both early and late delaminating NBs. Indicating that Cas can act as a *pdm* repressor outside of its normal late expression boundaries, the temporally misexpressed Cas significantly reduced Pdm-1 expression when compared with wild-type embryos stained under identical conditions (Fig. 8). The inability of the ectopic Cas to completely suppress Pdm-1 expression may be caused by the lack of sufficient levels of Cas necessary to completely override endogenous *pdm* transactivators. Ectopic Cas had no effect on the dynamics or levels of Hb expression (data not shown).

drf and I-POU require *cas* for full CNS expression

The role of Cas control over the *pdm* genes raises the possibility that it may regulate expressions of other POU genes. To test this, we compared the expression domains of Cas and Drf, and examined Drf expression in *cas*[−] embryos. In addition to its established role in midline glia and tracheal development, Drf is also expressed in a subset of NB progeny in both the developing brain and

Figure 5. Loss of *hb* disrupts axon fasciculation and triggers ectopic Pdm-1 expression in early developing neuroblast sublineages. (A,B) Correct formation/organization of both longitudinal and commissural axon fascicle tracks requires *hb* function. Shown are dorsal views of dissected wild-type (A) and *hb*⁴ null mutant (B) stage 13 ventral cords immunostained in whole-mount with an axon marker, the BP102 mAb (anterior is up). Note the lack of longitudinal connectives and missing or disorganized commissures in the *hb*[−] ventral cord. (C–F) In *hb*[−] mutant embryos, early delaminating CNS NBs (S1 and S2 waves) fail to terminate Pdm-1 expression. Comparisons between wild-type (C) and the *hb*⁴ null allele (D) late stage 12 embryos reveal increased numbers of NB sublineages expressing Pdm-1 in all CNS ganglia of the *hb* mutant. Shown are dissected flattened fillets of whole-mount immunostained embryos (anterior is up). Transverse sections through abdominal segments of late stage 9 (E) and stage 12 (F) *hb*⁴ embryos reveal ectopic Pdm-1 expression in early delaminating NBs and in their sublineages. Note the increased numbers and thicker dorsal/ventral field of Pdm-1-positive cells in the stage 12 mutant ventral cord (F) when compared with the Pdm-1 immunoreactive cells present in a similar transverse section from a wild-type embryo shown in Fig. 3H. (G,H) Shown are a transverse section through the second abdominal segment (G) and a dissected fillet (H) both from late stage 12 *hb*⁴ embryos immunostained with Cas antibodies. In *hb*⁴ mutants, late developing NB sublineages express Cas. When compared with wild-type Cas expression patterns, however, its onset is delayed in certain sublineages (data not shown) and the overall position of Cas-expressing cells is altered in *hb*[−] disrupted neuromeres. In *hb*[−] embryos, Cas-positive late sublineages are still found on the outer ventral surfaces of the ventral cord neuromeres.

ventral cord (Anderson et al. 1995). We find that many Cas-expressing NB sublineages also express Drf (Fig. 9A). Thus, it appears that Cas does not repress Drf expression. On the contrary, a marked reduction in late-lineage Drf expression was observed in *cas*[−] embryos (Fig. 9B), suggesting Cas either directly or indirectly plays a role in activating and/or sustaining Drf expression in these sublineages. To test if Cas could ectopically activate *drf* outside of its endogenous expression boundaries, we examined Drf expression in the *prospero.GAL4/UAS.cas* embryos and found that ectopic Cas had no effect on Drf expression (data not shown).

In situ localization of *I-POU* mRNA in wild-type, *cas*[−] and *prospero.GAL4/UAS.cas* embryos yielded findings similar to *drf*. In the absence of *cas* function, *I-POU* expression is lost in a subset of ventral cord cells (Fig. 9C,D), but ectopic Cas has no effect on the *I-POU* wild-type expression pattern (data not shown). At this time, we do not know if Cas is a direct activator of *drf* and/or *I-POU*. The data indicates, however, that if Cas is playing a direct activator role, it most likely requires cofactors that are not expressed outside of its normal domain.

Discussion

The principal finding of this study is that the zinc finger proteins Hb and Cas act in a cooperative, nonoverlapping manner to control POU gene expression during *Drosophila* CNS development. By silencing *pdm* expression in early and late NB sublineages, Hb and Cas establish three pan-CNS compartments whose cellular constitu-

ents are marked by the expression of either Hb, Pdm, or Cas. Optical sections through stage 13 and older embryos simultaneously immunostained for Hb, Pdm-1, and Cas reveal that these expression compartments encompass most, if not all, NB lineages. We hypothesize that these sequentially formed transcription factor expression domains represent temporal branch points in the regulatory network controlling cell identities. The fact that cells within a given domain share the expression of one of these transcription factors suggests that subsets of cells in a given expression domain may also have overlapping repertoires of transcriptionally active genes that ultimately produce shared or interrelated cellular functions. The loss of axon fascicle organization in *hb*[−] embryos may represent an example of this shared transcription factor interdependence. Descendants of early NB sublineages most likely depend on Hb regulation for correct expression of axon pathfinding signals and/or the machinery to accurately interpret these guidance cues. The transcription factor expression domains reported here bear notable similarity to temporally orchestrated events that occur during mammalian brain development. For example, in the developing cerebral cortex of mammals, neurons are generated in an orderly layered inside-out progression (for review, see McConnell 1988). Populations of cortical neurons within a given layer share birthdates, coexpress certain POU and/or homeobox genes (Frantz et al. 1994a,b), have common functional properties, and display similar connectivity (for review, see McConnell 1995).

Although a detailed understanding of the mecha-

Figure 6. Loss of *castor* results in ectopic Pdm-1 and Pdm-2 expression in late developing neuroblast sublineages. (A,B) Comparisons of Pdm-1 immunostaining in wild-type (A) and *cas*[−] null (B) stage 13 ventral cords reveals ectopic *pdm* expression in late-stage *cas*[−] NBs and in their progeny. Pdm-1 misexpression is particularly evident in the ventral-lateral regions of the *cas*[−] ventral cord. In wild-type ventral cords, these ventral-lateral areas are predominantly made up of Cas-expressing sublineages (see Fig. 4C). Shown are the third thoracic through third abdominal neuromeres of early stage 13 embryos (anterior is up). (C,D) Ectopic Pdm-1 expression is also evident in late developing cephalic lobe NB sublineages. Shown are dorsal views of wild-type (C) and *cas*[−] (D) stage 13 Pdm-1 immunostained embryos (anterior is up). (E,F) Ni-enhanced, Pdm-2 immunostaining of wild-type (E) and *cas*[−] (F) embryos also reveals ectopic Pdm-2 expression in late-stage *cas*[−] cephalic lobe NBs. Note, focal planes are similar to C and D.

nism(s) that generate the *Drosophila* CNS expression domains awaits further analysis, the tightly choreographed NB expressions of Hb, Pdm, and Cas suggest temporally integrated processes participate in their formation. Clonal analysis of ventral cord NB lineages has revealed that many early delaminating NBs produce lineages that span most of the ventral cord's dorsal/ventral axis (Pro-

kop and Technau 1994; Buenzow and Holmgren 1995; Bossing et al. 1996). For example, the NB5-2, one of the first NBs to delaminate, generates a ventral-dorsal column of 17–26 cells (Bossing et al. 1996). The dynamics of Hb, Pdm-1, and Cas expression in NBs indicates that many of the early S1 and S2 delaminating NBs may sequentially express all three and thereby produce lineages spanning all three compartments. Two such ventral cord candidates are the early NB5-2s and NB7-4s. Shortly after their delamination, during early stage 9, they activate Hb expression (Fig. 2A, inset), whereas later, after several rounds of GMC divisions, they activate Cas expression (Fig. 2C, inset; Cui and Doe 1995). The fact that we detect NBs coexpressing Hb/Pdm-1 or Pdm-1/Cas, but never Hb/Cas, further suggests that at least some of the early NBs make the Hb → Pdm → Cas transition. Not all NBs undergo these transitions, however. This is particularly evident in NBs that enter the proliferative zone during later delamination waves. For example, the first ventral cord NBs to express Cas, the S3 NB6-1s, activate Cas shortly after delaminating from the ectoderm and do not express Hb (Fig. 2B, inset).

Previous studies reveal Hb to be a highly versatile regulator of gene expression (for review, see Rivera-Pomar and Jackle 1996). The dynamic expression of Hb in early sublineages may allow for *pdm* expression at specific stages during sublineage development. For example, the *pdm* genes are known to collaborate in the S2 NB4-2 → GMC-1 → RP2 neuron lineage by specifying GMC-1 identity (Bhat et al. 1995; Yeo et al. 1995). Interestingly, *pdm* expression is detected only in GMC-1 and not in its NB. The lack of Hb/Pdm-1 overlap in GMCs or in their progeny suggests Hb may dynamically regulate *pdm* expression by first silencing their NB expression and then, in absentia, permitting *pdm* reactivation in the GMC-1. Recent studies on the annelid ortholog of *hb*, *Lzf2*, also suggest that it may play a temporal regulatory role during leech nervous system development (Savage and Shankland 1996).

Our studies reveal that late developing sublineages rely on *cas* to both insulate cell-fate programs and to secure the expression of factors that likely play key roles in their cell identity decisions. Cas carries out the first of these regulatory roles by selectively silencing the expression of *pdm* genes. This is evidenced by the fact that late developing NB sublineages exhibit sustained, ectopic Pdm-1 and Pdm-2 expression in *cas*[−] embryos, *pdm-1* enhancer(s) are activated in *cas*[−] late NBs; and in contrast to its repressor role, *cas* is required for full-expression of *Drf* and *I-POU*. The process of reinforcing specific cell fates by silencing the expression of regulators that dictate alternative fates is one that is employed during many developmental decisions. For example, shortly after NB commitment, the pan-neural zinc finger protein Scratch promotes neural development by silencing regulators that dictate mesoderm cell fates (Roark et al. 1995). Like *scratch*, *hb* and *cas* participate in cell fate branch points positioned downstream of the initial proneural and neurogenic events.

Our efforts to characterize Cas DNA-binding specific-

Figure 7. Cas silences *pdm-1* neuroblast enhancer(s). (A,B) Whole-mount in-situ mRNA localizations in transformant embryos shows that a 5.3-kb fragment of *pdm-1* regulatory DNA can activate the divergently transcribed reporter genes, *mini-white* (A) and *GAL4* (B), in a manner similar to endogenous cellular blastoderm *pdm-1* expression (anterior, left; see Fig. 7G and Materials and Methods for P-element vector details). Note, *pdm* cellular blastoderm expression boundaries are set by Hb repression (Lloyd and Sakonju 1991; Cockerill et al. 1993). (C–F) In situ hybridizations performed on wild-type (C,E) and *cas*⁻ (D,F) transformant embryos reveal that the *mini-white* (C,D) and *GAL4* (E,F) reporter genes, are activated in *cas*⁻ CNS NBs. Shown are dorsal views of stage 13 cephalic lobes (anterior, up; focal plane passes through the dorsal surface of the cephalic lobes). (G) The *pdm-1* regulatory DNA/reporter gene P-element construct showing locations (solid ovals) of 32 potential Cas/Hb DNA-binding sites. These sites share 8 of 10 bp with the consensus Hb-binding site and all contain the core A/T-rich sequence (see Fig. 1C for consensus DNA-binding sequences). Core recognition sequences (upper strand) for sites 6 and 12 are shown plus a mutated site (m12) used to test Cas-DNA binding sequence-specificity (see H). Both sites were selected because they match known Hb recognition sites (Stanojevic et al. 1989; Treisman and Desplan 1989). Dashes in m12 represent same bases as in the wild-type site. The *pdm-1* embryonic transcriptional start

site is shown as a rightward-facing arrow. (H) Electrophoretic mobility-shift assays show that Cas binds specifically to sites in *pdm-1* regulatory DNA. Shown are gel-shift assays with 30-bp dsDNA fragments matching either Hb recognition site 6 or 12. Increasing concentrations (5- and 50-fold) of cold competitor DNA (6 or 12) reduce labeled DNA–Cas complexes, whereas the mutated fragment (m12) fails to bind Cas or compete (5- and 50-fold) with wild-type 12 DNA–Cas binding. Note, no recombinant Cas was added to the samples run in lanes marked –.

ity led to the unexpected observation that it selectively binds to DNA recognized by Hb. Although nothing is known about the high-resolution details of how Hb or Cas interact with DNA, comparisons drawn from data compiled on other C₂-H₂ finger-DNA complexes may be informative. Crystallographic analyses of different protein-DNA complexes reveal that not all fingers dock with DNA in the same way; hence, no simple code for base-pair specificity exists (Fairall et al. 1993; Pavletich et al. 1993; Elrod-Erickson et al. 1996). All finger-DNA structures resolved thus far, however, show that within a finger's α -helical reading head, two to four residues play key roles in conferring DNA-binding specificity. Secondary structure predictions of the Cas finger domain indicate only the first and third of its TFIIIA-like fingers contain α -helices. The relative positions of these α -helices and their predicted lengths in the fingers agree with helices in previously analyzed finger-DNA complexes. *cas* evolutionary comparisons identify these α -helices as

having the highest conservation (100% identity between *Drosophila melanogaster* and *Drosophila virilis*; R. Kambadur, C. Stivers, and W.F. Odenwald, unpubl.). Interestingly, optimal alignment of Cas and Hb fingers reveal that the first and third α -helices of Cas share the highest homology with the corresponding α -helices of Hb (33%/66% identity/similarity for the first and 27%/80% for the third). Although speculative, their shared DNA-binding preferences may, in part, be caused by the shared residues found in these predicted reading heads. Outside of their zinc fingers, Hb and Cas show no obvious sequence similarities. This may indicate that they interact with different cofactors and thus modulate the same target genes in similar or different ways depending on the presence or absence of specific ancillary factors. Future studies aimed at comparing the regulatory functions of these finger proteins will enhance our understanding of the genetic pathways leading to neuronal diversity.

Materials and methods

Drosophila P-element transformants and stocks

Standard *Drosophila* husbandry procedures were used (Ashburner 1989). Ectopic expression of *cas* in all NB lineages was accomplished by use of the *GAL4-UAS* strategy (Brand and Perrimon 1993). The P[*prospero.GAL4*] transformant line was kindly provided by F. Matsuzaki (NIN, Tokyo, Japan). A *UAS.cas* transgene was constructed with a 2.4-kb *Vent* polymerase (New England Biolabs) amplified *cas* cDNA fragment, containing only the *cas* ORF, inserted into the *Eco*RI site of the pUAST vector to produce P[UAS.*cas*]. The orientation and reading frame of the *cas* sequence was confirmed by DNA-sequence analysis. Germ-line transformants were isolated by standard techniques with the *yw*^{67c23} stock (Spradling 1986). Phenotypic analysis of *cas* mutants was performed on the *cas*⁻ *H23A* alleles, $\Delta 1$ and $\Delta 3$, generated by imperfect excisions of the P[*HlacZ*] vector (Mellerick et al. 1992). For identification of homozygous *cas*⁻ embryos, these recessive lethal alleles were balanced over the third chromosome *TM3,Sb,P[ftz/lacZ]* (Hiromi et al. 1985; Nambu et al. 1990). To examine the effect of a *cas* null background on *pdm-1* cis-regulatory DNA/reporter gene expression, a P[*pdm-1.GAL4*] reporter vector was constructed. The 2.8-kb *KpnI-NotI* fragment from pGATB (Brand and Perrimon 1993) containing the *GAL4* coding sequence was subcloned into the pCaSpeR4 vector polylinker and a 5.3-kb *Eco*RI fragment from the *pdm-1* promoter region was inserted into the *Eco*RI site of the pCaSpeR4 polylinker. Germ-line transformants were prepared and a P[*pdm-1.GAL4*] located on the third chromosome (for construct map, see Fig. 7G) was mobilized with $\Delta 2-3$ as a genomic source of transposase (Robertson et al. 1988). Second chromosome jumps were saved, made homozygous, and selected for *mini-white/GAL4* expression that matched endogenous *pdm-1* expression patterns. Males from one of these lines (no. 11), containing the third chromosome *Ser/TM-3,Sb,P[ftz/lacZ]* balancers, were mated to virgins with the following second and third chromosomes: *CyO/Sco; $\Delta 1,H23A/TM-3,Sb,P[ftz/lacZ]$* . Sibling crosses were carried out with flies possessing the following chromosomes: 11P[*pdm-1A.GAL4*]/*CyO; $\Delta 1,H23A/TM-3,Sb P[ftz/lacZ]$* . Progeny, homozygous for the 11P[*pdm-1A.Gal4*] second chromosome, were crossed to establish a stock containing the third chromosomes: *$\Delta 1,H23A/TM-3,Sb P[ftz/lacZ]$* . Phenotypic analysis of loss of *hb* function was carried out with the *hb*⁴ null allele. Balancer chromosomes used above are described in Lindsley and Zimm (1992).

mRNA localization

Localization of mRNA by whole-mount in situ hybridization of fixed embryos was performed with digoxigenin-labeled DNA probes according to Tautz and Pfeifle (1989). Probes were generated by the random primer method with the Genius Kit (Boehringer Mannheim). Templates for probes used included: a 1.6-kb *pdm-1* cDNA fragment; a *mini-white* 2-kb *SacI* fragment from pCaSpeR vector (Pirrotta 1988); an *I-POU* 4.2-kb *Eco*RI-*HindIII* genomic fragment (kindly provided by S. Certel and W. Johnson, University of Iowa, Iowa City); and a *GAL4* 3.1-kb *Bam*HI fragment from the pGATB vector (Brand and Perrimon 1993).

Recombinant Cas protein production and purification

A bacterially expressed, histidine-tagged Cas fusion protein was prepared by the pET expression system (Studier et al. 1990) according to the manufacturer's protocol (Novagen). Briefly, an overnight *Escherichia coli* culture [strain BL21 (DE3)] harboring

Figure 8. Targeted misexpression of Cas in all neuroblast lineages reduces Pdm-1 expression. (A) Activation of a *UAS.cas* transgene by the *prospero.GAL4* driver results in Cas misexpression in most, if not all, NB lineages in both the CNS and in the PNS (arrows). Shown is a transverse section through an abdominal segment of a stage 13 *prospero.GAL4/UAS.cas* transformant embryo immunostained in whole-mount with Cas antibodies (ventral is up). Scale bar equals 25 μ m. (C,D) Comparisons of ventral cord fillets from Pdm-1 immunostained wild-type (C) and *prospero.GAL4/UAS.cas* (D) stage 13 embryos, processed under identical conditions, reveal that ectopic Cas reduces, but does not eliminate, Pdm-1 expression. Shown are the second and third thoracic neuromeres plus abdominal neuromeres 1–5. The focal plane for both C and D passes through the layers containing the highest density of Pdm-1-positive cells.

the pET 16b plasmid containing a *cas* cDNA *Bam*HI restriction fragment (coding for the 239 amino acid zinc finger repeat plus 196 and 120 amino- and carboxy-terminal residues, respectively) was diluted and grown up to an OD of 0.8 in 1 liter of LB medium plus 50 μ g/ml ampicillin. Induction of the recombinant Cas was accomplished by the addition of 1 mM IPTG to the culture and continued incubation for 2 hr. Bacteria were collected by centrifugation, resuspended in 40 ml of lysis buffer [6 M guanidine hydrochloride, 20 mM Tris (pH 8.1), 5 mM 2-mercaptoethanol], cooled to 4°C, and sonicated for 5 min with intermittent cooling. The lysate was centrifuged at 10,000g for 30 min and the supernatant was saved for Cas purification by the Ni-agarose affinity protocol of Shirakata et al. (1993). Column fractions containing Cas were pooled; dialyzed against two changes of 50 mM Tris-HCl (pH 8.0) containing 200 mM KCl,

Figure 9. Both *Drf* and *I-POU* require *cas* for full-expression. (A,B) Confocal views of Cas (red) and *Drf* (green) simultaneous localizations in whole-mount immunostained stage 13 wild-type (A) and *cas*⁻ (B) embryos shows: (1) many late developing NB sublineages coexpress *Drf* and Cas (yellow cells in A); and (2) loss of *cas* results in reduced numbers of *Drf* expressing NB sublineages (B). Shown are lateral views with focal planes passing through the right cephalic lobes (dorsal is to the right, anterior is up). Note, *Drf* expression in the epidermis of *cas*⁻ embryos is unaltered. Also note, the absence of Cas immunostaining in *cas*⁻ embryos confirms the specificity of the Cas antisera. (C,D) In situ mRNA localization of *I-POU* expression in wild-type (C) and *cas*⁻ (D) embryos show that *cas* function is necessary for full *I-POU* expression. Shown are ventral focal plane views of dissected stage 13 ventral cords (third thoracic and first three abdominal segments; anterior, up). Note, *I-POU* expression in the medial-lateral cluster (arrowhead in C) is significantly diminished in the *cas*⁻ ventral cord.

10% glycerol overnight; aliquoted; and stored at -80°C . A detailed description of this protocol is available upon request.

Cas polyclonal antibody production

Mouse anti-Cas sera was raised against the recombinant protein by use of the protocols described in the Harlow and Lane antibody manual (1988). Briefly, BALB/c female adult mice were injected subcutaneously at multiple sites with 50 μg of purified recombinant protein in 2 ml of PBS/RIBI adjuvant system [1:1 (vol/vol) RIBI Immunochem Research Inc.]. Fifteen days later, the mice were boosted with the same inoculum and 1 month after the initial inoculation, a third boost was administered by intraperitoneally injecting ~ 50 μg of Cas protein/mouse. Fifteen days after this final boost, mice were exanimated by bleeding out, and serum was stored at -80°C for immunohistochemistry. To raise polyclonal anti-Cas antibodies in rabbits, New Zealand

white virgin females were given multiple subcutaneous injections of the purified recombinant Cas (500 μg /rabbit) mixed with the RIBI adjuvant (1:1). Rabbits were boosted with the same inoculation 30 and 45 days later. A final boost containing just Cas (500 μg /rabbit) was administered on day 60, and 10 days later the rabbits were terminally bled, their serum collected, aliquoted, and stored at -80°C . The specificity of both anti-Cas antibodies was confirmed by the absence of immunostaining in *cas* null embryos (see Fig. 8B; data not shown).

DNA-binding studies and molecular biology techniques

The consensus DNA-binding site for Cas was determined from an unbiased set of degenerate oligonucleotides by the cyclic amplification and selection of targets (CASTing) protocol of Wright et al. (1991). Preparation of the double stranded 75-mer oligonucleotides, recombinant Cas-DNA binding conditions, and immuno-affinity selection were performed according to their methods with the following exceptions. The complete sequence of the selected/Cas-bound DNA fragments is available upon request. Approximately 200 ng of the recombinant Cas was used for each binding reaction that was carried out in 50 mM Tris-HCl (pH 8.0), 400 mM KCl, 5 mM MgCl₂, 2 mM 2-mercaptoethanol, 10% glycerol, and 10 μM ZnCl₂. Protein-DNA complexes were immuno-selected by incubating with rabbit anti-Cas serum (final dilution 1:500) for 30 min at 4°C followed by the addition of protein A-coated magnetic beads (Dyna). Prior to their use, protein A beads were preincubated with 0.2% BSA in phosphate buffered saline (pH 7.4), 0.05% triton X-100 (PBT). Protein A-antibody complexes were recovered by a magnetic field. Following three washes with PBT, the beads were resuspended in 50 μl of $1\times$ PCR buffer and a 30- μl aliquot of this mixture was denatured at 95°C and subjected to 20 PCR cycles (92°C for 1 min, 65°C for 1 min, and 72°C for 1 min with a final extension time of 10 min). A 10- μl sample of amplified DNA was used to initiate the next CASTing cycle. CASTing cycles of 2, 3, and 4 were subjected to 15, 10 and 9 cycles of PCR, respectively. After four cycles of CASTing, 1 μg of amplified DNA was used as a template to generate a radiolabeled probe for band shifts by incorporating [^{32}P]dCTP during three cycles of PCR in the absence of cold dCTP by use of the forward and reverse primers as described by Wright (1991). Electrophoretic mobility-shift gel retardation assays with 200 ng of the purified recombinant Cas were performed as in Odenwald et al. (1989) with the following modifications. Prior to their use, probes were purified by electrophoresis through 8% polyacrylamide TBE gels by use of standard procedures (Sambrook et al. 1989). A final concentration of 400 mM KCl was used in the protein-DNA-binding reactions. Following electrophoresis of protein-DNA complexes, shifted bands were excised from the gel and the probe eluted into 200 μl of PBS by vigorous shaking of gel pieces at 37°C for 5 hr. A 25- μl aliquot of the eluted DNA was used as a template for the next round of PCR amplification. After an additional CASTing cycle with band-shift as the selection, amplified fragments were directly cloned into the pCR II vector (Invitrogen) and sequenced by the chain-termination sequencing method (Sanger et al. 1977). For antibody supershift assays, protein-DNA complexes were incubated with the rabbit anti-Cas (final dilution 1:500) for an additional 30 min at 4°C prior to electrophoresis. To test the binding specificity of Cas, 5- and 50-fold excesses (as determined by optical density measurements) of unlabeled cold DNA fragments were mixed with labeled probes prior to the addition of the recombinant Cas. The immunoprecipitation of Cas bound *pdm-1* genomic fragments was as follows: upstream *pdm-1* DNA genomic fragments were end-labeled with [^{32}P]ATP and T4 polynucleotide kinase ac-

cording to standard protocols (Sambrook et al. 1989). Labeled DNA fragments were purified by use of SR 200 Microspin columns (Pharmacia), and ~1 ng of the end-labeled fragments were added to binding buffer (same as the CASTing buffer) and incubated with 200 ng of the recombinant Cas on ice for 30 min. Protein-DNA complexes were immunoprecipitated as above and resolved by electrophoresis through standard agarose gels. DNA sequencing of the *pdm-1* regulatory DNA was accomplished by standard chain-termination procedures. The complete sequence of *pdm-1* DNA fragments used in the gel-shift assays is available upon request.

Immunocytochemistry

The antibody staining protocol was based on a procedure developed by N. Patel (1994), by use of the Vectastain ABC second antibody avidin/biotin HRP visualization reagents (Vector Labs) for light microscopy. For immunofluorescent confocal microscopy, FITC-, Rho-, Cy2-, or Cy5-conjugated secondary antibodies (Jackson Labs) were used. Detailed protocols are available upon request. To diminish background staining, the mouse and rabbit anti-Cas sera (1:50 dilutions) were preabsorbed with embryos (an overnight collection) for 48 hr at 4°C with one change of embryos. Final dilutions for the anti-Cas mouse and rabbit sera were 1:500 and 1:5000, respectively. Purified rabbit anti-Pdm-1 and Pdm-2 antibodies (Cockerill et al. 1993) were used at 1:25 and 1:10 dilutions, respectively. To enhance the Pdm-2 immunostaining, NiCl₂ and CoCl₂ were added to the diaminobenzidine chromogen solution according to Kania et al. (1990). Rat anti-Drf serum (kindly provided by M. Anderson and W. Johnson, University of Iowa) was preabsorbed as above and used at a final dilution of 1:3000. The rat anti-Hb (a gift from P. Macdonald) was also preabsorbed and used at a final dilution of 1:15000. The BP102 mouse mAb (a gift from Nipam Patel) was used at a final dilution of 1:20. *cas*⁻ embryos were identified by the absence of *lacZ* expression from the balancer TM-3,Sb,P[*ftz/lacZ*] chromosome with either rabbit anti-β-gal (Cappel, 1:2000) or mouse anti-β-gal Mab (Promega, 1:2000). Stained embryos were mounted in 70% glycerol for observation, and in some cases, flattened embryo fillets or dissected ventral cords were prepared with fine needles (Hamilton, cat. no. 90033). Embryos were also sectioned following whole-mount immunohistochemistry by first post-fixing in 2% glutaraldehyde in PBS and then individually encasing the embryos in albumin plugs to facilitate handling during the plastic embedding (Mellerick et al. 1992). Mounted embryos or dissected fillets were photographed on a Nikon Optiphot microscope with DIC/Nomarski optics. Immunofluorescent images were collected by confocal microscopy on a Zeiss LSM 410 equipped with a krypton/argon laser.

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Note added in proof

The GenBank accession no. for the genomic DNA containing *pdm-1 cis*-regulatory sequences is AF035264.

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Ravi Kambadur, Keita Koizumi, Chad Stivers, et al.

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