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The *miti-mere* and *pdm1* Genes Collaborate during Specification of the RP2/sib Lineage in *Drosophila* Neurogenesis

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We have investigated (i) the role of *pdm1*, a *Drosophila* POU gene, during the elaboration of the GMC-1→RP2/sib lineage and (ii) the functional relationship between *pdm1* and the closely linked second POU gene, *miti-mere*, in this lineage. We show that deletion of *pdm1* causes a partially penetrant GMC-1 defect, while deletion of both *miti* and *pdm1* results in a fully penetrant defect. This GMC-1 defect in *miti*$^-$ and *pdm1*$^-$ embryos can be rescued by the *pdm1* or *miti* transgene. Rescue is observed only when these genes are expressed at the time of GMC-1 formation. Overexpression of *pdm1* or *miti* well after GMC-1 is formed results in the duplication of RP2 and/or sib cells. Our results indicate that both genes are required for the normal development of this lineage and that the two collaborate during the specification of GMC-1 identity.

The central nervous system (CNS) of the *Drosophila* embryo is generated by a specialized group of progenitor cells called the neuroblast stem cells (NBs). The NBs are initially selected from among the cells of the neuroepithelium through the action of the proneural genes. Once an NB is formed, its neighboring ectodermal cells are prevented from becoming NBs by neurogenic genes involving cell-cell interactions (5, 6, 11, 16). Most of the NBs function much like stem cells, undergoing a series of asymmetric cell divisions producing a chain of ganglion mother cells (GMCs). These GMCs divide once, giving rise to cells that subsequently differentiate into neurons. When the differentiation of embryonic CNS is complete, each hemisegment is thought to consist of about 250 highly specific and distinct neurons.

To understand how a small number of NBs generate a diverse array of highly specialized neurons, it is necessary to dissect the mechanisms involved in cell fate specification at each step in the elaboration of neuronal lineages. We have begun to explore this problem of sequential cell fate specification by examining the development of one particular motoneuron lineage, NB4-2→GMC-1→RP2/sib. This lineage has been well characterized, and a number of genes such as *wingless*, *gooseberry*, *patched*, *prospero*, *fushi tarazu* (*ftz*), and *even-skipped* (*eve*) are known to be required for its proper elaboration (7, 12, 14, 18, 22). *wingless*, for instance, is thought to function in the initial specification of NB4-2 identity, while some of these other genes are required in GMC-1 (e.g., *prospero*) or later (*eve* and *ftz*). Another gene which appears to function in the RP2 lineage is the POU gene *miti-mere* (*miti*) (2), previously known as *pdm2* or *dPOU28* (3, 10). Two lines of evidence implicate *miti* in the development of the RP2/sib lineage. First, when a truncated miti protein, Δ*miti*, containing the DNA-binding POU domain but lacking the N-terminal activation domain, was expressed during neurogenesis, it behaved in a dominant negative manner, mimicking a loss-of-function mutation at *miti*, and interfered with the specification of the RP2 lineage (2). Second, when a full-length miti protein was misexpressed or overexpressed, it resulted in the duplication of the RP2/sib lineage (21).

Located distally about 50 kb from *miti* toward the telomere is another POU gene, *pdm1* (3, 8, 10). The two genes *pdm1* and *miti* show 87% identity in the DNA-binding POU-specific and POU homeodomains. Within the POU-specific domain there is a single amino acid difference, while there are five amino acids differences in helices 1 and 2 of the POU homeodomain (3, 10). Hence, it is possible that the miti and pdm1 proteins bind to the same DNA sequences, perhaps with somewhat different specificities, or preferentially bind to related but slightly different DNA sequences. While the two have extensive similarities in the C terminus, the N-terminal activation domains of *miti* and *pdm1* are quite different, and consequently the two proteins might be expected to interact with a different set of cofactors.

The close linkage of *miti* and *pdm1* and the high degree of similarity in the DNA-binding domains raise questions about the functional relationship between these two POU genes; however, nothing is known about the role of the *pdm1* gene, particularly during neurogenesis. To address these problems, we have examined whether *pdm1* has any role in the elaboration of the GMC-1→RP2/sib lineage. In this report, we show that *pdm1* is expressed in a pattern identical to that of *miti* during the development of this lineage and, like *miti*, is required for the specification of the GMC-1 identity. However, loss of function for *pdm1* results only in a partially penetrant GMC-1 defect (absence of *eve*-positive GMC-1→RP2/sib cells); we observe a fully penetrant lineage defect only when both *miti* and *pdm1* are deleted. This fully penetrant lineage defect can be rescued by *hsp70* transgenes expressing either the miti or the pdm1 protein just at the time of GMC-1 formation. Finally, overexpression of *pdm1* (and *miti*; see also reference 21) in a wild-type background has a different effect: it induces the duplication of RP2 and/or sib cells. Significantly, the optimal timings for rescue and duplication are different; duplication events are observed well after the GMC-1 cell has been formed. Taken together, our results indicate that these two POU genes have an unusual relationship; though they appear to have similar functions, they must collaborate in order to properly specify GMC-1 identity.

MATERIALS AND METHODS

Fly stocks. The transgenic lines carrying either *pdm1* or *miti* and *pdm2* have been previously described and shown to express the encoded proteins ubiqui-

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tously (8). The deficiencies *Proxless* (*Prl*) and *paired* (*prd*) *1.7* were obtained from the Indiana Stock Center. The breakpoints of these deletions have been described previously (2, 8, 17). These deficiency chromosomes were balanced with a LacZ-marked "blue" *CyO* balancer.

Staging of embryos. The embryos were staged as described by Wieschaus and Nusslein-Volhard (20) at room temperature (~22°C). We also used the development of two other lineages, the GMCs for U neurons and aCC/pCC neurons in relation to the development of the GMC-1→RP2/sib cells, for determining the developmental stage.

Immunostaining. Immunostaining with antibodies was performed as described previously (2). A polyclonal *eve* antibody was from Manfred Frasch. The *pdm1* antibody used is directed against the N-terminus portion of the protein and is *pdm1* specific (8). To detect differentiated RP2 motoneuron in embryos older than 13 h, a monoclonal antibody (MAb) against a membrane protein, 22C10 (a gift from the laboratory of C. Goodman), was used. For double staining with *eve* and *pdm1* antibodies, an *eve* MAb (from Nipam Patel) was used. For double labeling with *eve* and *ftz*, the polyclonal *eve* antibody and a MAb against *ftz* (gift from Ian Duncan) were used. In all double labeling experiments, the detection was done by confocal microscopy.

Heat shock and rescue experiments. For the overexpression of *pdm1* and *miti* transgenes, embryos were heat shocked as described previously (2). Briefly, embryos were hand picked during the cleavage stage, aged at 22°C for various lengths of time, and heat shocked at 37°C for specified durations by immersion in halocarbon oil preheated to 37°C. Embryos were allowed to develop for various lengths of time before fixing and staining with various antibodies. For rescuing, the RP2 lineage defect in *prd 1.7*, either the *pdm1* transgene or the *miti* transgene under the control of the *hsp70* promoter was introduced into the *prd 1.7* background. Early cleavage-stage embryos were hand picked and aged for different durations before the heat shock. The heat shock was performed for 25 min at 37°C as described above. Following heat shock, embryos were aged for 2 h, fixed, and stained with *eve* or double stained with *eve* and *ftz*. Control embryos in these heat shock experiments did not show any duplication of the RP2 lineage.

RESULTS

Expression of *pdm1* in the NB4-2→GMC-1→RP2/sib lineage. The parental NB for the RP2/sib lineage is NB4-2; this row 4 NB delaminates from the neuroepithelium during stage 9 of embryogenesis as an S2 NB (~5 h of development at 22°C). Shortly thereafter, NB4-2 begins a series of asymmetric divisions producing several distinct GMCs. The first of these is GMC-1 (Fig. 1A). This GMC becomes *eve* positive during early stage 11 (~7 h); about 1 h later (~8 h at 22°C), it divides to give one cell which subsequently differentiates into the RP2 motoneuron and innervates muscle 2 and a second, sib cell whose ultimate fate is not known. In a previous study, we found that expression of the *miti* POU gene can first be detected in this lineage at about 6.5 h, or very soon after the GMC-1 cell is formed (2). The level of miti protein peaks at ~7 h and then begins to drop. By contrast, over this same time period the level of eve protein, which is very low when the GMC-1 cell is first formed, increases. When the GMC-1 cell divides, the remaining miti protein appears to be asymmetrically distributed into the newly formed RP2 and sib cells, with higher levels in RP2 cell and lower levels in the sib cell (2). eve protein also shows a similar asymmetric distribution in RP2 and sib cells (Fig. 1A). Eventually miti protein disappears from both RP2 and sib cells, while eve disappears only from the sib cell.

To ascertain whether the sister of *miti*, *pdm1*, might also play a role in elaboration of the RP2/sib lineage, we first used a *pdm1* antibody (8) to determine whether *pdm1* is expressed in the GMC-1→RP2/sib lineage. To help identify cells in this lineage, we used an antibody directed against *eve*. Figure 2 shows a confocal analysis of the CNS, double stained with *eve* and *pdm1* antibodies, at two different time points, approximately 6.5 and 8 h of development (at 22°C). At the earlier stage, the newly formed GMC-1 cell should be located just above the cluster of *eve*-positive aCC/pCC and U GMCs. Although an *eve*-positive cell at the position of GMC-1 is not detected in either the left or right hemisegment (Fig. 2a; the approximate position is marked by an arrow), a *pdm1*-positive cell is found in the right hemisegment at the position expected

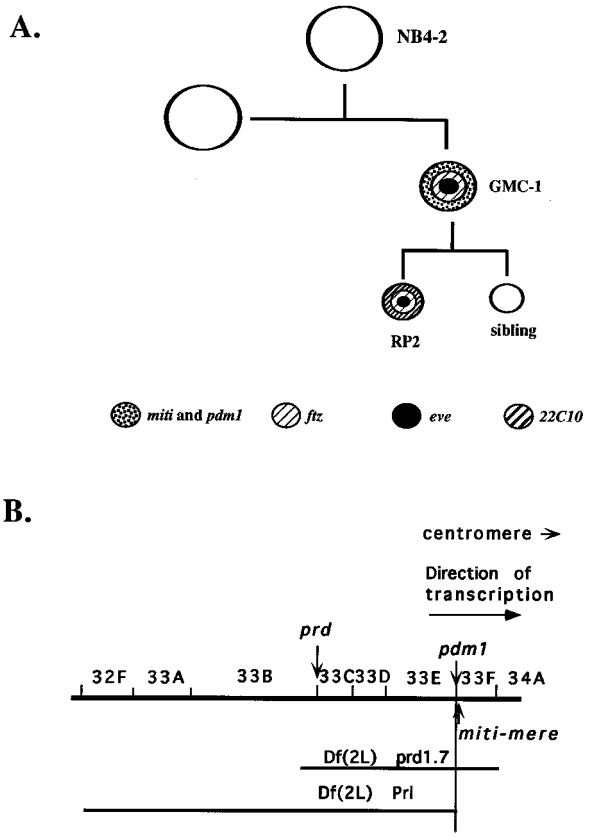

FIG. 1. (A) The NB4-2→GMC-1→RP2/sib lineage. The first GMC produced from NB4-2 gives rise to the RP2 motoneuron and its sibling cell. At or very soon after the GMC-1 cell is first formed, it begins to express the *miti* (2) and *pdm1* (this report) genes. Shortly thereafter, *eve* and *ftz* expression is activated, while the level of *miti* and *pdm1* begins to drop. All four proteins are still present in GMC-1 when it divides to form the RP2 and sib cells; however, the four proteins appear to be asymmetrically distributed into the two daughters, with higher levels in the RP2 cell than in the sib cell. While *eve* and *ftz* continue to be expressed in RP2 as it differentiates into the motoneuron, *pdm1* and *miti* are no longer detected. The fully differentiated motoneuron expresses an additional marker, a membrane protein recognized by MAb 22C10. The ultimate fate of the sib cell is not known; however, if it survives, it does not appear to express any of these marker proteins. (B) Chromosomal map of the 33F region on the left arm of the second chromosome. Both *miti* and *pdm1* genes map to the cytological interval of 33F1/2 and are transcribed in the same direction toward the centromere. The distance between *miti* and *pdm1* is about 50 kb. Also shown are the approximate positions of breakpoints for the deficiencies *prd 1.7* and *Prl*.

for GMC-1 (Fig. 2b, arrow). That this *pdm1*-positive cell is likely to correspond to GMC-1 is suggested by the finding that the *pdm1*-positive cell at this position (relative to the aCC/pCC and U GMCs; large arrow) becomes *eve* positive at a slightly later point in development. These results suggest that *pdm1*, like *miti*, is probably activated in the RP2/sib lineage shortly after GMC-1 is formed.

As the GMC-1 cells age, the level of pdm1 protein appears to drop, while the level of eve protein increases. Figures 2d to f show eve and pdm1 proteins in the CNS of an ~8-h embryo, just around the time when the GMC-1 cells begin to divide. Typically, the newly formed RP2 cell appears to be larger (hemisegment on the left in Fig. 2d) and have higher levels of eve and miti proteins than the sib cell, which is smaller and usually appears to have lower levels of these two proteins. A similar pattern is also observed for pdm1 protein (hemisegment on the left in Fig. 2e and f): the RP2 cell is larger and appears to contain more protein (arrow) than the smaller sib

FIG. 2. Expression of *pdm1* in wild-type GMC-1→RP2/sib cells. Wild-type embryos were doubly stained with *eve* and *pdm1* antibodies, and the staining pattern was analyzed by confocal microscopy. The photographs shown represent the sum of several images sectioned across the CNS. Anterior end is up. The thick lines on the top and bottom indicate the midline. (a) *eve* staining pattern in a ~6.5-h embryo. At this stage, *eve* is barely detectable in GMC-1 (position is marked by an arrow above the GMCs for U neurons [open arrow] and aCC/pCC neurons [thick arrow]) in this hemisegment. (b) The same field visualized for pdm1 protein. A cell at the position expected for GMC-1 shows strong expression of *pdm1* (arrow). (c) Merging of images from panels a and b. (d to f) *eve* and *pdm1* staining patterns in a slightly older embryo (~8 h). In the hemisegment on the left (d), the GMC-1 cell has already divided to form RP2 (arrow) and sib (thin arrow) above the U neurons (open arrow) and aCC/pCC cells (thick arrow). As is typically observed, the RP2 cell is larger and shows stronger *eve* staining than the sib cell. (e) Partitioning of pdm1 into RP2 and sib cells. Typically the larger RP2 cell appears to have somewhat more pdm1 protein than the smaller sib cell. The merged image (f) demonstrates that pdm1 protein is present in both the RP2 and sib cells. In the hemisegment on the right (d to f), the GMC-1 cell appears to be in the process of dividing into RP2 and sib cells.

cell (thin arrow). eve protein then persists in the RP2 cell during its subsequent differentiation into a neuron (Fig. 1a), while it gradually disappears from the sib cell. However, pdm1 protein disappears from both cells soon after they are formed (data not shown).

Overexpression of *pdm1* causes duplication of the RP2/sib lineage. In a previous study, Yang et al. (21) showed that overexpression of miti protein from a heat shock *miti* cDNA results in the formation of two RP2 and two sib cells instead of just one of each. If *pdm1* also functions in the elaboration of the RP2/sib lineage, then overexpression of the pdm1 protein might produce similar duplications. To test this possibility, we briefly heat shocked animals carrying either an *hsp70:pdm1* transgene or, as a control, an *hsp70:miti* transgene around 7 h of development (at 22°C). The heat-shocked embryos were then allowed to develop for an additional 2 to 3 or 6 h and stained with *eve* antibody. As illustrated in Fig. 3B (thick arrow), we observed a duplication of the *eve*-positive RP2 neurons in the CNS of 13-h embryos. The duplicated RP2 neurons could be detected in 1 to 12 hemisegments in about 72% of the *hsp70:pdm1* embryos (Table 1). A similar percentage (71%) of embryos having duplicated RP2 neurons in two to six hemisegments (Fig. 3C; Table 1) was observed with the *miti* transgene.

To confirm that the RP2 neuron is duplicated by ectopic *pdm1* (or *miti*) expression, we stained the 13-h heat-shocked embryos with MAb 22C10, which is direct against a membrane protein. RP2 is one of the neurons which expresses this antigen on its cell body and axonal tract membranes (Fig. 3D). MAb 22C10 also stains other RP neurons, such as RP1, RP3, and RP4 (Fig. 3D). As was found with *eve* staining, we observe hemisegments in the heat-shocked *pdm1* or *miti* embryos which have two 22C10-positive RP2 neurons instead of one. This duplication is shown for *pdm1* in Fig. 3E. In addition to the duplication of the RP2 neurons, we occasionally observed hemisegments in the heat-shocked *pdm1* embryos which are missing RP1, RP3, or RP4 neurons, as judged by 22C10 staining (Fig. 3E). However, it should be noted that the duplication of RP2 does not appear to depend on the loss of one or more of the other RP neurons; most hemisegments that have duplicated RP2 neurons also have the other three RP neurons, while hemisegments which lack RP1, RP3, or RP4 typically have only a single RP2 neuron. Similar results were also observed for heat shocked *miti* transgenic embryos as well (data not shown). These results indicate that the ectopic expression of *pdm1* or *miti* can cause CNS defects other than the duplication of RP2.

TABLE 1. Effects of a brief heat shock of *miti* and *pdm1* transgenic embryos on GMC-1→RP2/sib lineage[a]

Characteristic	*pdm1*			*miti*		
	No. of hemisegments with	% of embryos with	Total no. of embryos counted	No. of hemisegments with	No. of embryos with	Total no. of embryos counted
3 cells	1–3	12	42	1–2	11	37
4 cells	1–10	69	42	1–7	65	37
Duplicated RP2 neurons	1–12	72	32	2–6	71	30

[a] The hemisegments with three cells and four cells are counted from the same set of embryos. The number of hemisegments with three cells include (i) two weakly staining (for *eve*) and/or smaller cells and one strongly staining and/or larger cell and (ii) two larger and/or strongly staining cells and one smaller and/or weakly staining cell (see Fig. 3).

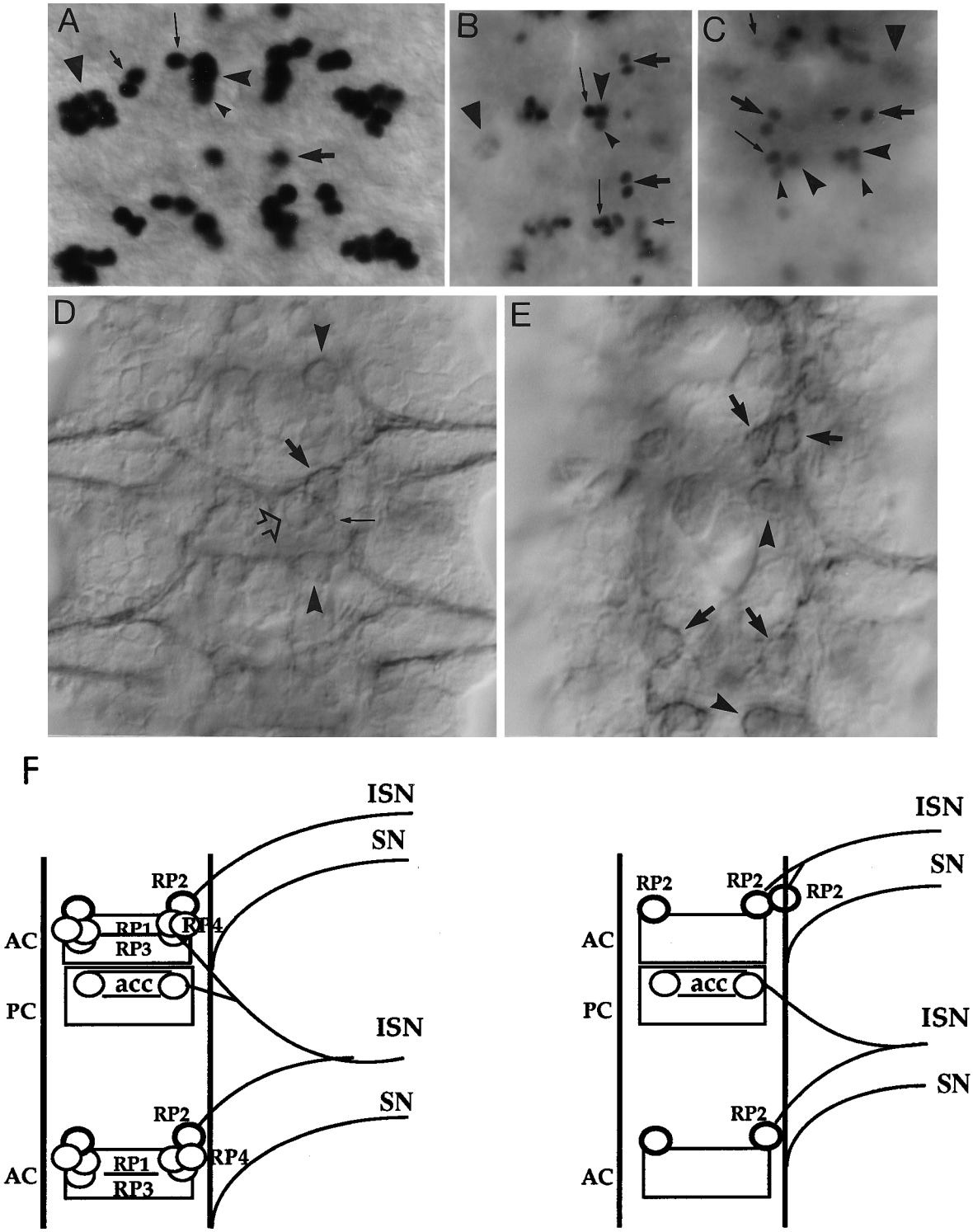

FIG. 3. Overexpression of *pdm1* or *miti* causes duplication of the RP2 neuron. Hand-picked embryos aged for ~7 h were heat shocked at 37°C for 25 min. Following heat shock, embryos were allowed to develop for 6 h at 22°C before staining with *eve* and MAb 22C10. Anterior end is up. In panels A to C, the embryos were stained with *eve* antibody. (A) Control embryo. (B and C) Heat-shocked *hsp70:pdm1* and *hsp70:miti* transgenic embryos. In the control embryo, *eve* antibody stains the RP2 (thick arrow), aCC (large caret), pCC (small caret), U (thin arrow), CQ (small arrow), and EL (arrowhead) neurons. In the heat-shocked *hsp70:pdm1* embryo (B), the two hemisegments on the right each have two *eve*-positive RP2 neurons instead of one (marked by thick arrow). In each of the hemisegments on the left, only one RP2 neuron is present; the RP2 cell in the lower hemisegment is not in the focal plane. In the heat-shocked *hsp70:miti* embryo (C), both the upper left and right hemisegments contain two *eve*-positive RP2 neurons, while only one RP2 motoneuron is present in the lower left and right hemisegments, and these are slightly out of the focal plane. Other *eve*-positive lineages do not appear to be affected in these transgenic embryos. (D and E) Control and heat-shocked *hsp70:pdm1* embryos stained with MAb 22C10. MAb 22C10 (D) stains the RP2 cell body (thick arrow) as well as RP1 (empty arrow), RP4 (thin arrow), RP3 (out of focus in this photomicrograph; panel F), aCC (caret), and several other neurons. A duplicated RP2 neuron can be seen in the upper right hemisegment in the *hsp70:pdm1* embryo in panel E, and the cell bodies of these two neurons are indicated by the thick arrows. The lower right hemisegment has only a single RP2 neuron. Also indicated by carets in this panel are two aCC neurons. Several other abnormalities are evident in this embryo, including a disruption in the organization of the commissures and longitudinal connectives and apparently missing RP1, RP3, and RP4 neurons. Very similar results were also observed for heat-shocked *miti* transgenic embryos (not shown). (F) Organization of some of the neurons in the CNS as revealed by MAb 22C10 in the control (left) and heat shocked *hsp70:pdm1* (right) embryos. ISN, intersegmental or ipsilateral nerve bundle (RP2, aCC, and other neurons contribute to this bundle); SN, segmental nerve bundle; AC, anterior commissure; PC, posterior commissure.

We next determined whether the elaboration of the RP2/sib lineage is sensitive to elevated levels of *pdm1* at the same time in development as it is to *miti*. Yang et al. (21) showed that the *hsp70*:*miti* embryos had to be heat shocked after the GMC-1 cell is formed in order to for a lineage duplication to be observed. By contrast, earlier heat shocks, before the parental NB4-2 cell divides, or later, after the GMC-1 cell has divided to produce RP2 and sib cells, do not induce lineage duplications. To determine when *pdm1* induces lineage duplications, staged transgenic embryo collections were heat shocked at different time points in the interval between 1 and 9 h after egg lay and then allowed to develop until 13 h. Duplication of the *eve*-positive RP2 cells was observed only when the *hsp70*:*pdm1* transgenic embryos were heat shocked around 7 h of development (at 22°C). We found that a similar timing was required to induce duplications with the *miti* transgene (21). These results are consistent with the idea that the GMC-1 cell is sensitive to elevated levels of *pdm1* and *miti*, while RP2 and sib cells are not.

To provide additional evidence that the extra *eve*-positive RP2 neuron observed in the 13-h CNS is likely due to an earlier duplication of GMC-1, we examined the development of the RP2/sib lineage in embryos heat shocked at ~7 h of development. As would be expected for a GMC-1 duplication, we found that a subset of the *pdm1* or *miti* transgenic embryos at 1 h after heat shock had two instead of one *eve*-positive cell in the location of GMC-1 (data not shown). When slightly older (8- to 8.5-h) embryos were examined, nearly 70% (Table 1) had between 1 and 10 hemisegments with four cells (two RP2 and two sib cells) for *hsp70*:*pdm1* transgenic embryos and 1 to 7 hemisegments for *miti* embryos (Fig. 4c [*pdm1* embryo] and f [*miti* embryo]).

Duplication of the sib or RP2 cell but not both could also be observed after overexpression of *pdm1* or *miti*. To explain the duplication of the RP2 and sib cells, Yang et al. (21) proposed that elevated levels of *miti* cause the GMC-1 cell to produced daughter cells which reiterate the GMC-1 fate instead of differentiating directly into RP2 and sib cells. Consistent with this idea, we found that four copies of the endogenous *miti* gene could also produce extra RP2 and sib cells (2). However, our result differed in one important respect from those of Yang et al. (21) (and the results described above for *pdm1*): we observed duplication of either the sib or the RP2 cell but not both (2). To explain this finding, we suggested that a twofold elevation in the level of *miti* is not sufficient to induce both daughters to reiterate the GMC-1 fate; instead, only one daughter reiterates the GMC-1 fate (ultimately producing an RP2 and a sib cell), while the other daughter assumes either an RP2 or a sib identity.

An obvious question is whether this partial reiteration of the GMC-1 lineage also occurs after heat shock induction of the *hsp70*:*pdm1* transgene or, for that matter, the *hsp70*:*miti* transgene. That it might occur is suggested by the fact that many of the hemisegments in heat-shocked *hsp70*:*pdm1* transgenic embryos show no apparent GMC-1 defect. It would appear that the level of *pdm1* is not sufficient and/or the timing of the heat shock is not appropriate to alter lineage development in these hemisegments. Hence, it is conceivable that in heat-shocked embryos, there may be hemisegments in which the GMC-1 cell has levels of *pdm1* sufficient to induce a partial but not a complete reiteration of the GMC-1 lineage. Consistent with this possibility, we observed hemisegments in heat-shocked *hsp70*:*pdm1* transgenic embryos with three, not four, *eve*-positive cells (Fig. 4; Table 1). Shown in Fig. 4a is the CNS of an 8- to 9-h embryo (that had been heat shocked ~7 h of development) in which one hemisegment has three *eve*-positive cells; one of these is larger and/or stains strongly for *eve* (arrow) and presumably corresponds to RP2, while the other two are smaller and or weakly staining (thin arrows) and presumably correspond to sib cells. The embryo in Fig. 4b also has hemisegments with three *eve*-positive cells; however, in this case two cells are larger and stain strongly for *eve* (presumed RP2 cells) and one cell is smaller and/or stains weakly for *eve* (presumed sib cell). These two types of lineage duplications were observed in about 12% of the embryos and are usually restricted to one to three hemisegments (Table 1).

The three-cell phenotype observed after overexpression of *pdm1* suggested that it would be of interest to determine whether this partial lineage reiteration is also observed when *miti* is expressed from the *hsp70* transgene. As illustrated in Fig. 4d and e and Table 1, hemisegments containing three instead of four *eve*-positive cells are observed after induction of a *hsp70*:*miti* transgene. As was the case for *pdm1*, this three-cell phenotype is infrequent (Table 1), and in most instances we observe four *eve*-positive cells in heat-shocked embryos (Fig. 4f; Table 1). While the percentage of *hsp70*:*miti* embryos showing RP2 duplications in this experiment is similar to that observed for *hsp70*:*pdm1*, the penetrance of the phenotype is lower, and typically fewer hemisegments show the lineage duplication. This probably reflects differences in the level of pdm1 and miti proteins expressed by these particular transgenes rather than differences in the properties of the two proteins. This possibility is suggested by the fact that in a second *hsp70*:*miti* transgenic line, we found RP2 duplications in as many as 13 hemisegments (data not shown) (see also below).

Both *pdm1* and *miti* are required for the normal specification of the GMC-1→RP2/sib lineage. The fact that *pdm1* and *miti* display very similar expression patterns and induce similar types of duplications in the RP2/sib lineage when overexpressed raises the possibility that these two POU genes are redundant. Alternatively, the two genes may perform collaborative or overlapping functions in the elaboration of the GMC-1→RP2/sib lineage. Our previous studies using a synthetic dominant negative Δ*miti* transgene suggested that *miti* was required to establish GMC-1 identity and that in the absence of a wild-type level of *miti* function, this cell and/or its progeny, RP2 and sib cells, are not formed or fail to develop properly. Hence, it was of interest to determine whether the loss of *pdm1* function also blocks the specification of the GMC-1→RP2/sib lineage. To test this, we examined the GMC-1→RP2/sib lineage in a deficiency, *Prl*, whose centromere-proximal breakpoint lies within the *pdm1* gene (Fig. 1B) and whose homozygotes have no pdm1 protein (8). While the *Prl* deficiency does not delete *miti*, it does remove the centromere-distal pair-rule gene *prd*. As a consequence of the *prd* lesion, alternate segments are missing in embryos homozygous for the *Prl* deletion, and development of the RP2/sib lineage can be examined only in those 14 hemisegments which are still present. In a previous study (2), we found that *eve*-positive cells from RP2/sib lineage could be detected in those segments which are unaffected by the *prd* lesion. However, as indicated in Fig. 5B (uppermost hemisegment, arrow), a more detailed analysis of the RP2/sib lineage in these remaining segments reveals that *Prl* has a partial defect in the elaboration of this lineage. While approximately 70% of the *Prl* homozygotes are missing the *eve*-positive cells from the RP2/sib lineage, the expressivity of this defect is quite low, and in most cases the lineage is missing in only one to two hemisegments (Table 2). A similar partially penetrant phenotype was also obtained with the dominant negative Δ*miti* transgene (2). A quite different result is observed for the deficiency *prd 1.7*, which removes both *miti* and

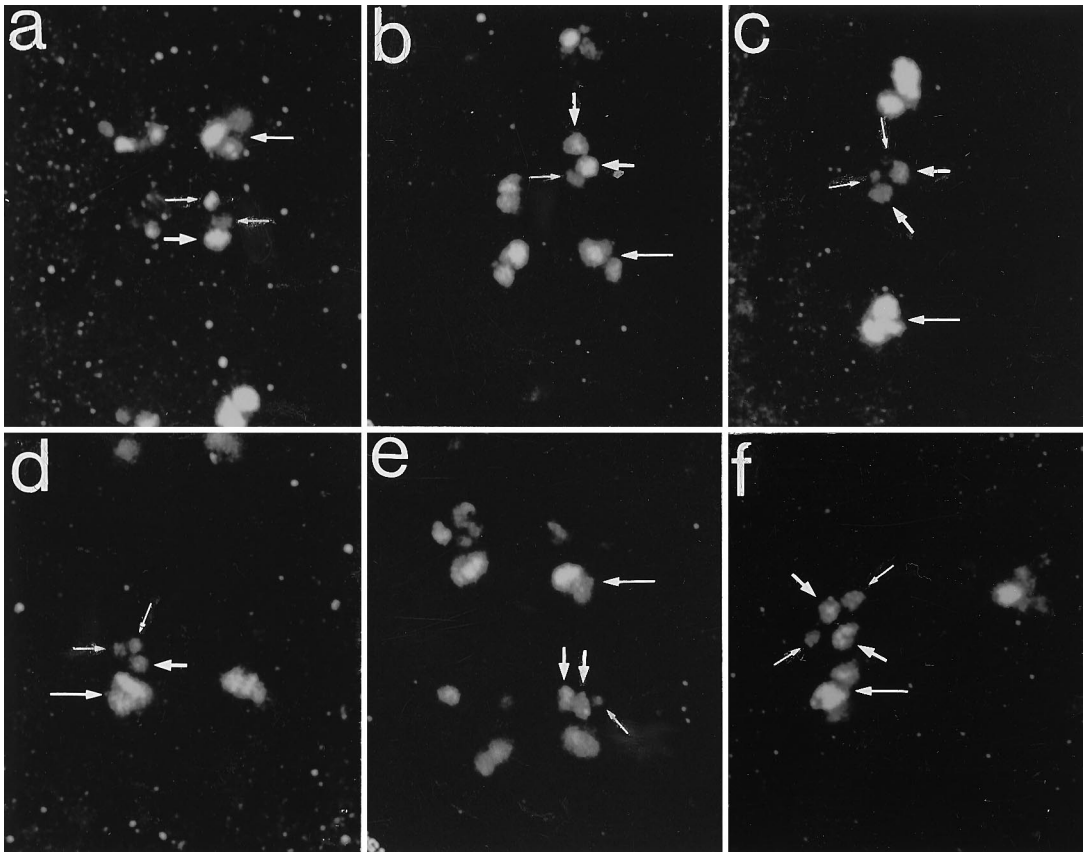

FIG. 4. Overexpression of *pdm1* and *miti* duplicates GMC-1. Hand-picked embryos aged for ~7 h were heat shocked at 37°C for 25 min. Following heat shock, embryos were allowed to develop for 2 to 3 h at 22°C before staining for *eve*. These embryos were examined with a confocal microscope. (a to c) *pdm1* transgenic embryos; (d to f) *miti* transgenic embryos. Anterior end is up; panels are dorsal views. (a and d) Instead of one RP2 cell and one sib cell, three cells are observed, two smaller and/or weakly staining cells (thin arrows) and one larger and/or strongly staining cell (arrow). (b and e) Three cells are also observed, but in this case two are larger and stain strongly for *eve* (arrows), while one is smaller and stains weakly for *eve* (thin arrow). (c and f) Four cells, two smaller cells with weaker *eve* expression (thin arrows) and two large cells with stronger *eve* expression (arrows), are observed. In panels a to c and d to f, the cluster of U and CQ cells are shown by a long arrow. aCC or pCC cells are not visible in these confocal sections. The images in these sections are slightly overcollected in order to visualize the weakly staining cells.

pdm1 as well as the *prd* gene (Fig. 1B). As shown in Fig. 5C, the RP2/sib lineage defect is completely penetrant (with full expressivity) in this deletion mutant—*eve*-positive cells from the RP2/sib lineage are absent in all remaining hemisegments (Table 2; Fig. 3F). These findings are consistent with the idea that loss-of-function mutations in both *pdm1* and *miti* may be required to disrupt the development in all hemisegments.

The results obtained for *prd 1.7* embryos that are transheterozygous for either the *Prl* deletion or a wild-type chromosome also suggest that both genes are required for the wild-type development of this lineage (Table 2). For instance, in contrast to the *Prl* homozygotes, all embryos which are transheterozygous for *prd 1.7* and *Prl* had hemisegments that exhibited RP2/sib lineage defects (Table 2). Thus, hemizygosity for *miti* appears to enhance the effects of loss of both copies of *pdm1* in the RP2 lineage. However, unlike the case for *prd 1.7* homozygotes, the RP2/sib lineage defect was not fully expressive in terms of the number of hemisegments affected in these transheterozygotes, and there were always hemisegments in which *eve*-positive RP2/sib cells could be observed (Fig. 5C; Table 2). Finally, as shown in Table 2, *prd 1.7* has a weak haplo insufficiency. When *prd 1.7* was outcrossed to a wild-type chromosome, we observed about 6.5% of *prd 1.7*/+ embryos with a missing *eve*-positive RP2 lineage. Since *Prl* does not show haplo insufficiency, this finding provides further evidence for the involvement of both *pdm1* and *miti* in the RP2/sib lineage specification.

The *prd 1.7* RP2/sib lineage phenotype is rescued by the *hsp70:pdm1* and *hsp70:miti* transgenes. *Prl* and *prd 1.7* are rather large deletions and hence might include genes other than *miti* and *pdm1* that are required for the proper elaboration of the RP2/sib lineage. If this were the case, the RP2/sib lineage defects evident in embryos carrying these deficiencies could be due to the deletion of these other genes rather than to the loss of *pdm1* or *miti*. Hence, it was important to determine whether the RP2/sib lineage defects evident in the deletion mutants could be rescued by the *pdm1* or *miti* transgene. For this purpose, we took advantage of the inducible transgenes in which *pdm1* or *miti* cDNAs are expressed under the control of the *hsp70* promoter.

In the first experiment, we tested whether the *hsp70:pdm1* transgene was able to rescue the fully penetrant defect in the RP2/sib lineage observed in homozygous *prd 1.7* embryos. Homozygous deficiency embryos carrying either one or two copies of the *hsp70:pdm1* transgene were heat shocked around the time of GMC-1 formation (see below), and the RP2/sib lineage was then examined by staining with *eve* antibody. The rescue of *eve*-positive cells in homozygous *prd 1.7* embryos after expression of *pdm1* is shown in Fig. 5D. As indicated in Table 3, over 40% of the *prd 1.7* homozygous embryos carrying a single copy

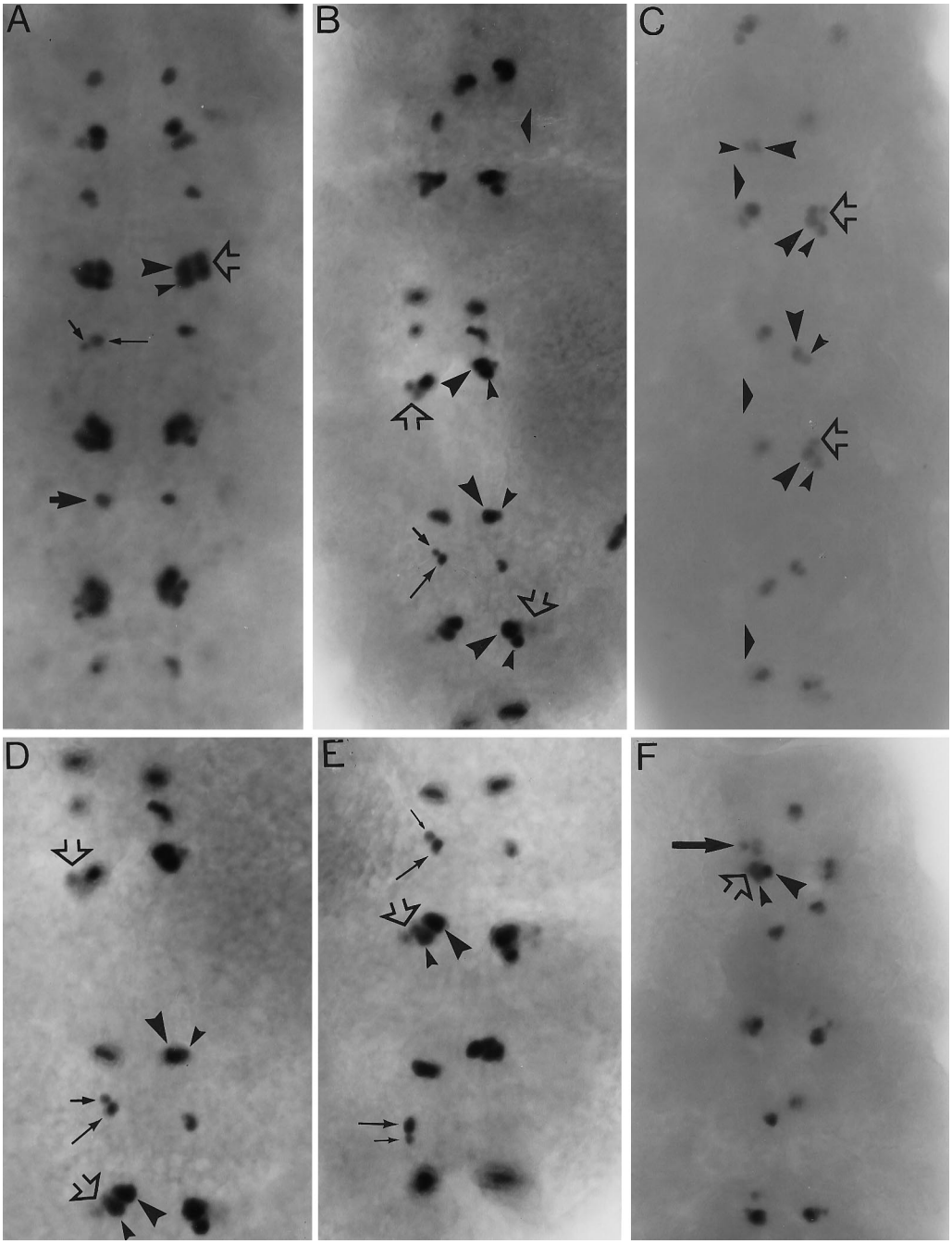

FIG. 5. The *eve*-positive RP2/sib lineage in *prd 1.7* homozygotes (*miti*$^{-}$; *pdm1*$^{-}$) can be rescued by either a *pdm1* or a *miti* transgene. Anterior end is up; panels are dorsal views. (A) A 7.5- to 8-h wild-type embryo. In all panels, cells are marked as follows: GMC-1, thick arrow; newly formed RP2, thin arrow; sib, short arrow; aCC and pCC, large and small carets, respectively; Us, open arrow. Note that the development of these lineages is not always synchronous even in adjacent hemisegments, and thus in some hemisegments only one cell (e.g., a GMC) instead of two (e.g., postmitotic neurons) are observed. (B) CNS of an embryo homozygous for the deficiency *Prl*, which deletes *prd* and *pdm1* but leaves *miti* intact. In this embryo, only one hemisegment has missing *eve*-positive RP2/sib cells (marked by an arrowhead, top hemisegment on the right). The absence of the RP2/sib and U neurons in alternate hemisegments is due to the *prd* mutation uncovered by this deficiency (Fig. 1). That the other *eve*-positive lineage cells deleted in this deficiency (and in *prd 1.7*) in alternate segments are the U neurons and not the aCC/pCC neurons is indicated by double staining for *eve* and *ftz* (shown in Fig. 6). U neurons are negative for *ftz*, while aCC and pCC cells and the GMC-1→RP2/sib cells are *ftz* positive. By contrast, the aCC and pCC neurons appear to be unaffected by the removal of the *prd* gene (in either *Prl* or *prd 1.7* deficiency embryos), and these neurons can be seen both just above and just below the cells from the RP2/sib lineage. (C) A 7.5 to 8-h homozygous *prd 1.7* deficiency embryo. This deficiency deletes *miti*, *pdm1*, and *prd*. No *eve*-positive GMC-1 or RP2/sib cells are observed in the hemisegments which are unaffected by *prd* deletion (expected positions of cells in this lineage; GMC-1, RP2 and sib are marked by arrowheads). However, the U neurons (open arrow) in the hemisegments unaffected by *prd*, and aCC and pCC neurons (carets) are still present in the *prd 1.7* embryos (just as is the case in *Prl* embryos). The *eve* staining pattern in the CNS of older deficiency embryos is often difficult to interpret because of the severe segmentation defects. (D to F) The rescue effects of *pdm1* (D) and *miti* (E and F) transgenes. Both *pdm1* and *miti* transgenes rescue the RP2 lineage in some hemisegments of *prd 1.7* deletion embryos. Duplication of the lineage in the *prd 1.7* background was also observed with the overexpression of *miti* (heavy arrow in panel F) or *pdm1* (not shown); however, the frequency of such duplication is very low.

TABLE 2. Frequency of penetrance of the RP2 lineage defect in deficiencies which removes *pdm1* or *pdm1* and *miti*[a]

Genotype	GMC-1→RP2/sib lineage defect	
	% of embryos	No. of hemisegments
Prl/*Prl*	70	1–6 (2)
prd 1.7/*prd 1.7*	100	14
Prl/*prd 1.7*	100	1–8 (3)
Prl/+	0	0
prd 1.7/+	6.5[b]	1–5 (2)

[a] Both *Prl* and *prd 1.7*, the two deficiencies which remove either *pdm1* or both *pdm1* and *miti*, also remove the *prd* gene (Fig. 1). The *prd* lesion enabled us to identify homozygous deletion mutants, since these embryos lack alternate segments. However, because of the *prd* segmentation defect in these deletion mutants, only a total of 14 hemisegments can be assayed for disruptions in the RP2/sib lineage. The average number of hemisegments affected is shown in parentheses.

[b] The strength of the *prd 1.7* haplo insufficiency shows some dependence on background. When *prd l.7* is outcrossed to other second chromosomes, we have observed both an enhancement and a suppression of this haplo insufficiency.

of the *hsp70*:*pdm1* transgene had at least one and in some cases as many as five hemisegments (those that are unaffected by the *prd* lesion of *prd 1.7*) with *eve*-positive RP2/sib cells. Moreover, rescue was observed in nearly twice the number of embryos (80%) when the homozygous mutants had two copies of the *hsp70*:*pdm1* transgene.

The *hsp70*:*miti* transgene also rescues the RP2/sib lineage defect in homozygous *prd 1.7* mutants to a *Prl*-like phenotype (Fig. 5E); however, as indicated in Table 3, *eve*-positive RP2/sib cells are observed only with two copies of the transgene. Even in this case, the percentage of rescued *prd 1.7* embryos is not much greater than that obtained with a single copy of the *hsp70*:*pdm1* transgene. As noted above, this difference between the rescue effects of *pdm1* and *miti* may reflect differences in the levels of two proteins produced by heat induction. In this regard, it may be significant that we rarely observe the duplication of the RP2 and sib cells in the *prd 1.7* background (solid arrow in Fig. 5F). We next determined whether the rescue of the RP2/sib defect in *prd 1.7* can be enhanced by the induction of both *miti* and *pdm1* transgenes. As indicated in Table 3, one copy of each POU gene is marginally more effective than two copies of *miti* but is less effective than two copies of *pdm1*.

To provide further evidence that the RP2 lineage defect in *prd 1.7* could be rescued by induction of either the *hsp70*:*pdm1* or *hsp70*:*miti* transgene, we double stained the heat-shocked transgenic *prd 1.7* embryos with *ftz* and *eve* antibodies. In the wild-type RP2 lineage, the *ftz* expression pattern is similar to *eve* pattern; it is first turned on in GMC-1, and when this cell divides, it appears to partition preferentially into the RP2 cell, while lower levels are found in the sib cell (Fig. 6a to c). *ftz* is also expressed in many other neuronal lineages (Fig. 6a to c); however, only some of these (e.g., aCC and pCC) are also *eve* positive (see also reference 13). Conversely, not all *eve*-positive neuronal lineages (e.g., U) express *ftz* (Fig. 6a to c). By double staining with *eve* and *ftz* antibodies, the RP2 lineage can be identified and distinguished from other nearby *eve*-positive neurons. We first examined the lineage in *prd 1.7* and *Prl* embryos. As shown for *prd 1.7* in Fig. 6d to f, cells from the RP2 lineage cannot be detected with either the *ftz* antibody or the *eve* antibody in these deficiency embryos. This finding provides further evidence that the GMC-1→RP2/sib lineage is not properly specified in embryos lacking both the *miti* and *pdm1* genes. By contrast, in *Prl* embryos which have the same defects in segmentation as *prd 1.7*, the RP2 lineage is present in most of the hemisegments unaffected by the removal of the pair-rule gene *prd*, and the cells from this lineage express both *eve* and *ftz* (Fig. 6g to i). There are, however, usually one or two hemisegments in *Prl* embryos which lack an *eve*-positive cell from the RP2 lineage (Table 2), and in these instances no *ftz* expression could be detected as well (data not shown). We next examined *eve* and *ftz* expression in *prd 1.7* embryos rescued by heat induction of either the *hsp70*:*miti* or *pdm1* transgene. As illustrated for *hsp70*:*miti* in Fig. 6j to l, the *eve*-positive cells induced by the transgene at the position expected for the RP2 lineage also express *ftz*. Similar results were obtained for *hsp70*:*pdm1*. The fact that the *eve*-positive cells formed after induction of the transgenes also express a second marker for the RP2/sib lineage provides additional evidence that *miti* and/or *pdm1* can rescue the lineage in *prd 1.7* embryos.

TABLE 3. Rescue of *miti* and *pdm1* deletion mutants with *miti* and *pdm1* transgenes[a]

Genotype	GMC-1→RP2/sib lineage rescue	
	% of embryos	No. of hemisegments
prd 1.7; *hsp70 pdm1* *prd 1.7*	45	1–5
prd 1.7; *hsp70 pdm1* *prd 1.7 hsp70 pdm1*	80	3–6
prd 1.7; *hsp70 miti* *prd 1.7*	0	0
prd 1.7; *hsp70 miti* *prd 1.7 hsp70 miti*	49	1–4
prd 1.7; hsp70 pdm1 *prd 1.7 hsp70 miti*	57	1–5

[a] In *prd 1.7* embryos, both *pdm1* and *miti* are deleted, and the RP2 lineage defect is observed in all hemisegments (not affected by the *prd* lesion) in all deletion mutant embryos. The ability of *pdm1* and *miti* transgenes to rescue this RP2/sib lineage defect (in the 14 hemisegments) was determined by scoring the appearance of *eve*-positive GMC-1 and/or RP2/sib cells.

The pdm1 and miti proteins are required during the time of GMC-1 formation. The experiments described above suggested that it might be possible to use these inducible transgenes to determine when in development the pdm1 and miti proteins are required for the proper elaboration of the GMC-1→RP2/sib lineage. Staged *prd 1.7* embryos carrying either the *hsp70*:*pdm1* or *hsp70*:*miti* transgene were heat shocked for 25 min at hourly intervals during development, and the rescue of the RP2/sib lineage was examined by *eve* antibody staining. As can be seen in Fig. 7, no rescue was observed when the two POU genes are induced during the first 4 h of development. A small percentage of the embryos show rescue when the transgenes are induced at 5 h of development, which is around the time when NB4 is formed. The percentage of rescued embryos for both transgenes shows a sharp peak at around 6 h, which is close to the time when NB4-2 divides to form GMC-1, and then drops dramatically at 7 h. This timing suggests that both POU proteins are required in the GMC-1→RP2/sib lineage at or just before the time when the GMC-1 cell is formed. Interestingly, this result is consistent with our analysis of the time when a dominant negative *hsp70*:Δ*miti* transgene exerts its maximum effect on the RP2/sib lineage (2). In this case, the disruption of the RP2/sib lineage by the inducible Δ*miti* transgene peaked at 6 h, dropping off sharply either earlier or later.

DISCUSSION

During evolution, gene duplication events provide in the short term a mechanism for boosting the level of critical proteins and in the long term the possibility that each of the

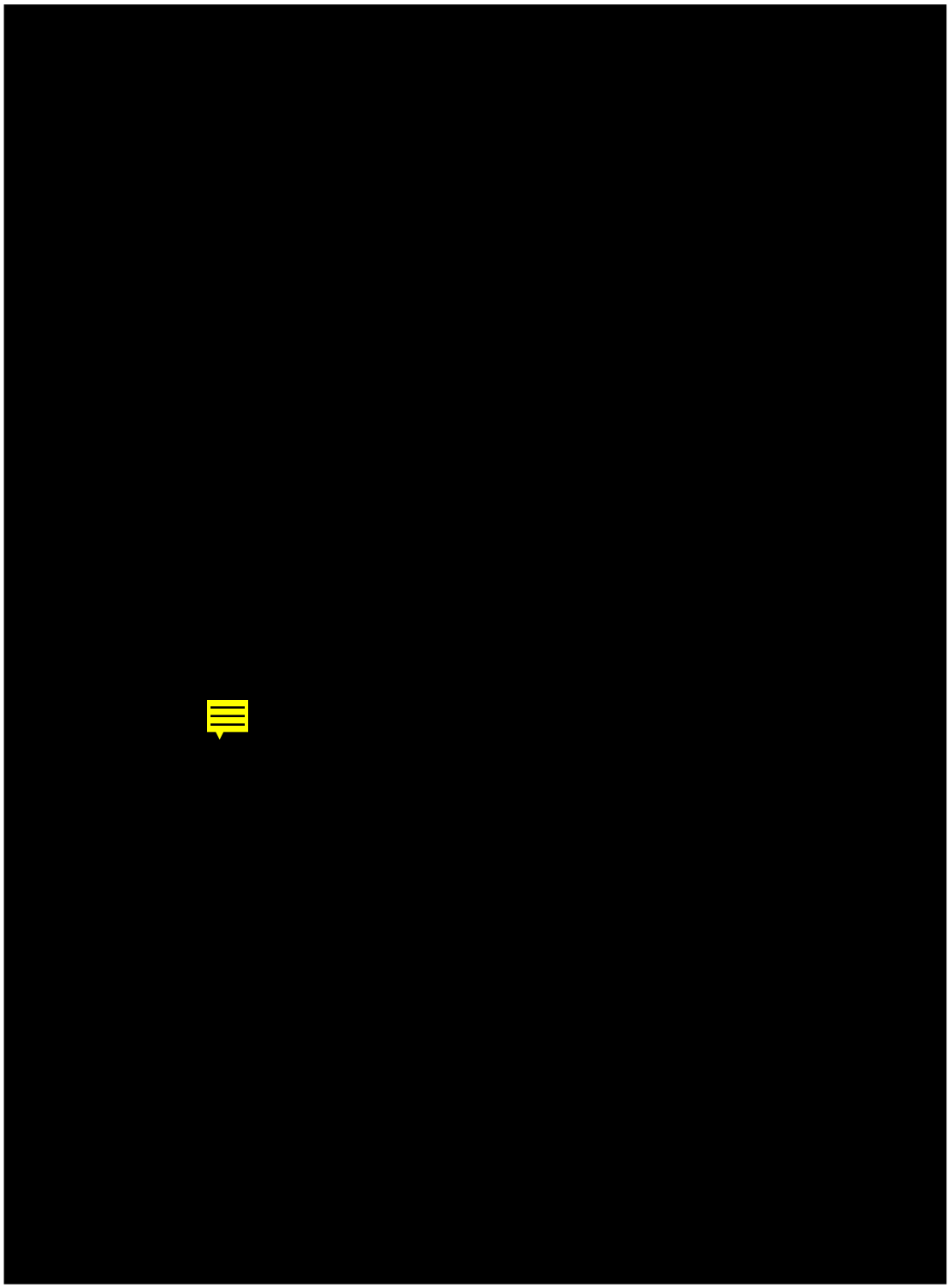

FIG. 6. The *eve*-positive cells rescued in *prd 1.7* embryos also express *ftz*. Shown are embryos double stained with *eve* (a, d, g, and j) and *ftz* (b, e, h, and k) antibodies. The *eve* and *ftz* images are merged in panels c, f, i, and l. The anterior end is up, and the midline is indicated by a line. (a to c) A wild-type embryo. In the left hemisegment, the RP2 and sib cells are indicated by thin long and thin short arrows, respectively. As evident from the merged image in panel c, these cells are *eve* positive and *ftz* positive. In the right hemisegment is a large GMC-1 cell (open arrow) which is *eve* positive (a). This cell is also *ftz* positive (b and c). The aCC and pCC cells which are located below the GMC-1 cell in the right hemisegment are indicated in panel a by a thick arrow and a small arrow. Both of these cells are also *ftz* positive (b and c). Finally, the U neurons are indicated by the curved arrow in panel a. These cells are *ftz* negative (b and c). (d to f) A *prd 1.7* homozygous embryo. In the top left and right hemisegments, the only visible *eve*-positive cells are aCC and pCC. These are also *ftz* positive (e and f). In the right hemisegment on the bottom, the *eve*-positive aCC, pCC, and U neurons can be seen in panel a (thick arrow, small arrow, and curved arrow, respectively). Of these, only the aCC and pCC neurons are also *ftz* positive (e and f). In the hemisegment on the bottom left, the U neurons are out of the focal plane, while the GMC for aCC and pCC neurons has not yet divided. Note that the *eve*-positive, *ftz*-positive RP2/sib lineage is not missing. (g to i) A *Prl* homozygous embryo. In the top left and right hemisegments, the RP2 and sib cells

are indicated by thin long and thin short arrows, respectively.These are *eve* and *ftz* positive (i). In the hemisegment on the bottom right are the cluster of aCC, pCC, and U neurons (indicated by thick arrow, short arrow, and curved arrow, respectively). (j to l) A *prd 1.7* embryo rescued by ectopic expression of an *hsp70*:*miti* transgene. In the hemisegment on the left is a GMC-1 cell (open arrow). This cell is *eve* positive (j) and *ftz* positive (k and l). It is located just above a cluster of aCC, pCC, and U neurons indicated by a thick arrow, a small arrow, and a curved arrow, respectively. In the hemisegment on the right (j) are *eve*-positive RP2 and sib cells (thin long and thin short arrows, respectively). Both cells are also *ftz* positive (k and l). In the hemisegment on the top right are aCC and pCC neurons (thick arrow and small arrow), while U neurons are missing because of the *prd* deletion (see also panels d to f for *prd 1.7* or panels g to i for *Prl*). On the top left is a GMC for aCC and pCC neurons that has not yet divided. It should also be noted that *ftz* is also expressed in the *eve*-positive cells that are rescued by ectopic expression of *pdm1* in *prd 1.7* deficiency embryos.

duplicated genes will acquire distinct and specialized functions. In *Drosophila melanogaster*, several developmental loci which contain two closely linked genes that encode highly homologous proteins and often exhibit somewhat similar or overlapping expression patterns have been identified. Some of the examples include *engrailed* (9), *zen* (19), and *gooseberry* (1, 4). While studies on the *zen* (*zen1* and *zen2*) and *engrailed* (*engrailed* and *invected*) loci have not yet elucidated the functional relationship between members in each of these loci, there is some evidence that the genes from the *gooseberry* locus, *gsb-distal* and *gsb-proximal*, may have distinct roles in the CNS development (15, 22). In the work described here, we have investigated the functional relationship between two closely linked POU genes, *pdm1* and *miti*, which may have originated by just such a gene duplication event.

We have focused on the roles of these two genes in the development of a specific neuronal lineage, NB4-2→GMC-1→RP2/sib. While previously published studies indicate that *miti* is required for the proper elaboration of this lineage, nothing was known about *pdm1*. In the studies reported here, we have first shown that *pdm1* displays a pattern of expression in the GMC-1→RP2/sib lineage that is quite similar to the *miti* pattern. Both genes are initially expressed just or very soon after NB4-2 undergoes its first asymmetric division to produce GMC-1. Both are present at very high levels in GMC-1 at the time when this cell first becomes *eve* positive. While the level of eve protein increases in GMC-1 during the interval before it divides, the levels of two POU proteins are down regulated. During the division of GMC-1, both proteins are asymmetrically segregated into the daughter cells, with higher levels in the presumptive RP2 cell and lower levels in the presumptive sib cell (see also reference 2).

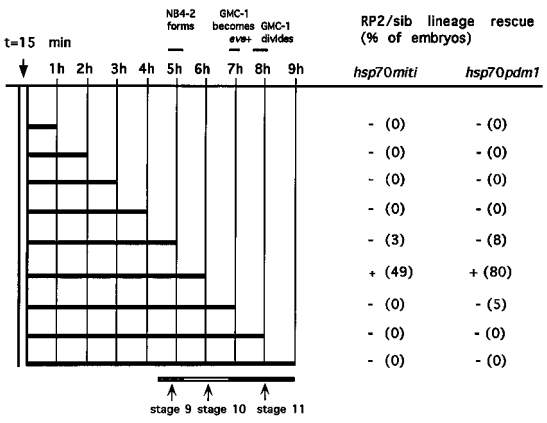

FIG. 7. The heat shock *miti* and *pdm1* transgenes rescue the RP2/sib lineage only during the formation of GMC-1. Embryos hand selected during the early cleavage stage were allowed to develop for various lengths of time at 22°C as shown by the vertical lines and heat shocked at 37°C for 25 min. After the embryos had aged for an appropriate period of time after egg lay, they were fixed and stained with *eve* antibody, and the percentage of *prd 1.7* homozygous embryos which have the GMC-1→RP2/sib cells was determined.

Our results argue that similarities in function underlie these similarities in expression pattern. The first are the effects of overexpression. For both *pdm1* and *miti*, overexpression leads to the duplication of RP2 and/or sib cells. Time course studies indicate that the lineage is sensitive to elevated levels of both miti and pdm1 proteins in the seventh hour of development and that the two genes exert their effects on GMC-1 and not on NB4-2 or RP2 and sib cells (see also reference 21). The second line of evidence comes from the effects of deletions on the specification of GMC-1. For instance, in *Prl*, which removes *pdm1* but leaves *miti* intact, the GMC-1→RP2/sib lineage defect is partially penetrant. Thus, only about 70% of the mutant embryos have detectable RP2/sib lineage defects, and these are typically restricted to only a subset of the hemisegments. A similar, partially penetrant phenotype was evident in experiments in which we used the dominant negative Δ*miti* transgene to mimic a *miti* loss-of-function mutation (2). In contrast, in *prd 1.7*, a deletion which lacks both *pdm1* and *miti*, the *eve*-positive GMC-1→RP2/sib lineage is completely absent. Taken together, these results argue that both genes are required and play a role in GMC-1→RP2/sib lineage. The third line of evidence for functional similarity comes from our rescue experiments. We have found that the RP2/sib lineage defect in embryos homozygous for the deletion lacking both *miti* and *pdm1* genes can be rescued by expression, from an *hsp70* promoter, of cDNAs corresponding to either gene. Moreover, time course experiments indicate that both genes are required in the lineage at the same point in development, namely, just as GMC-1 is formed from NB4-2.

Function of *miti* and *pdm1* in the RP2/sib lineage. What is the function of *pdm1* and *miti* in the GMC-1→RP2/sib lineage? A number of lines of evidence suggest that *pdm1* and *miti* play a crucial role in the initial specification of GMC-1. In previous studies, we found that the dominant negative protein expressed by an *hsp83*:Δ*miti* transgene interfered with the formation of GMC-1, and this *eve*-positive cell was either missing or behaved anomalously (2). Moreover, experiments with an inducible *hsp70*:Δ*miti* transgene indicated that the dominant negative protein had to be expressed at about the time when NB4-2 was dividing to produce GMC-1. Consistent with a requirement for *miti* and also *pdm1* at the time of GMC-1 formation are the results of the rescue experiments described here. Both the *hsp70*:*miti* and *hsp70*:*pdm1* transgenes must be induced just as the GMC-1 cell is being formed.

While the two POU genes appear to play an important role in the initial specification of the GMC-1 cell, both are down regulated as the cell progresses through the cell cycle and gets ready to divide to form RP2 and sib cells. That this down regulation may be important for the proper elaboration of the lineage is suggested by the results of overexpression. As we have shown here for *pdm1* and as Yang et al. (21) have shown previously for *miti*, overexpression of the proteins from *hsp70* transgenes in a wild-type background leads to the duplication of the RP2 and/or sib cells. Moreover, while rescue requires induction of the transgenes during GMC-1 formation, the optimal induction time for duplication events occurs after the

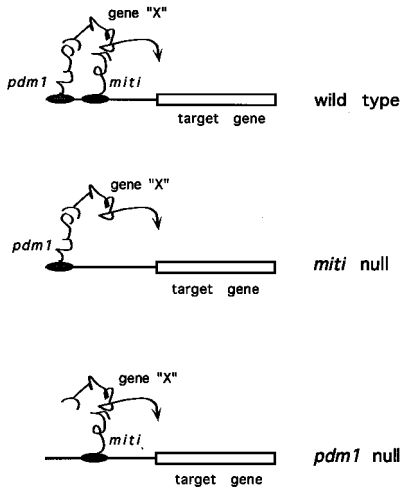

FIG. 8. A model to show how *miti* and *pdm1* genes might control GMC-1→RP2/sib lineage specification. In the wild type (A), *miti* and *pdm1* proteins bind to similar upstream octamer sequences of target genes. The two make contact with a third protein, "X," and establish a stable transcription initiation complex, which regulates the target gene expression. In *miti* (B) or *pdm1* (C) loss-of-function conditions, the initiation complex formed is less stable, leading to variable levels of target gene activation.

GMC-1 cell has already formed (approximately midway between GMC-1 formation and its subsequent division to give the RP2 and sib cells). These results suggest a model in which the levels of the two POU proteins must drop below a critical threshold at some point during the GMC-1 cell cycle (e.g., after replication) in order to initiate a developmental program that ultimately enables the GMC-1 daughter cells to assume new identities. When the level of one or the other POU protein is very high, this program is not initiated, and both daughters reiterate the GMC-1 fate. When the level of the POU proteins is elevated only a few fold, the program is triggered incorrectly—one daughter retains GMC-1 identity, while the other is able to assume a new identity.

Functional relationship between *pdm1* and *miti*. What is the functional relationship between *pdm1* or *miti* in the elaboration of the RP2/sib lineage? Our results indicate that both genes are required for the wild-type development of the RP2 lineage. A deletion which removes both genes completely eliminates the RP2 lineage, while a deletion which affects only one gene has a partially penetrant phenotype. Given these results and the results from the rescue experiments, the functional relationship between *pdm1* and *miti* can best be explained by two different models. In the first, the *miti* and *pdm1* genes would be functionally redundant; they would bind to the same target genes and interact with many of the same protein cofactors. The fact that both proteins are needed for wild-type development would simply reflect a quantitative requirement. In this scenario, the specification of the GMC-1 cell can proceed only when there is a certain threshold level of POU protein, and this level is very close to that produced by two copies of either one of the two genes. Hence, two different genes are required to ensure that the level of the POU proteins is well above this threshold. In the second model, there would be qualitative differences in the functional properties of the two proteins, and they would collaborate in the specification of GMC-1 (Fig. 8). This view is supported by the fact that only the DNA-binding POU domains show a high degree of sequence similarity, while there is little or no similarity elsewhere in the two proteins. Thus, though *pdm1* and *miti* could potentially recognize the same or an overlapping set of DNA sequence motifs, they could very well differ in the ability to interact with other proteins, and each may have domains that favor contacts with a different set of cofactors. A difference in cofactor specificity could be exploited when both POU proteins are bound to sites in a downstream target genes. Because each POU protein would contribute qualitatively different interaction domains, a transcriptional complex containing both might be more stable than a complex formed with only one (Fig. 8).

It seems likely that there may be some truth in both of these models. Arguing in favor of the additive model is the haplo insufficiency of *prd 1.7*. However, arguing in favor of the collaborative model is the fact that the *prd 1.7* deletion is only weakly haplo insufficient and the frequency of RP2/sib lineage defects is much less than that observed in *Prl* homozygotes. Moreover, hemizygosity for *miti* only marginally enhances the effect of *Prl* on the RP2/sib lineage. Hence, though gene dose may be important, there must also be significant qualitative difference between the two POU proteins that enable them to collaborate during GMC-1 specification (Fig. 8). In this context, it is interesting that while *miti* and *pdm1* appear to function together in the development of the RP2/sib lineage, a similar relationship is unlikely to exist in other CNS lineages. This conclusion is based on our finding that several neuronal lineages express *pdm1* or *miti* but not both. This observation raises the possibility that these two genes have assumed functionally distinct roles in the elaboration of these other CNS lineages.

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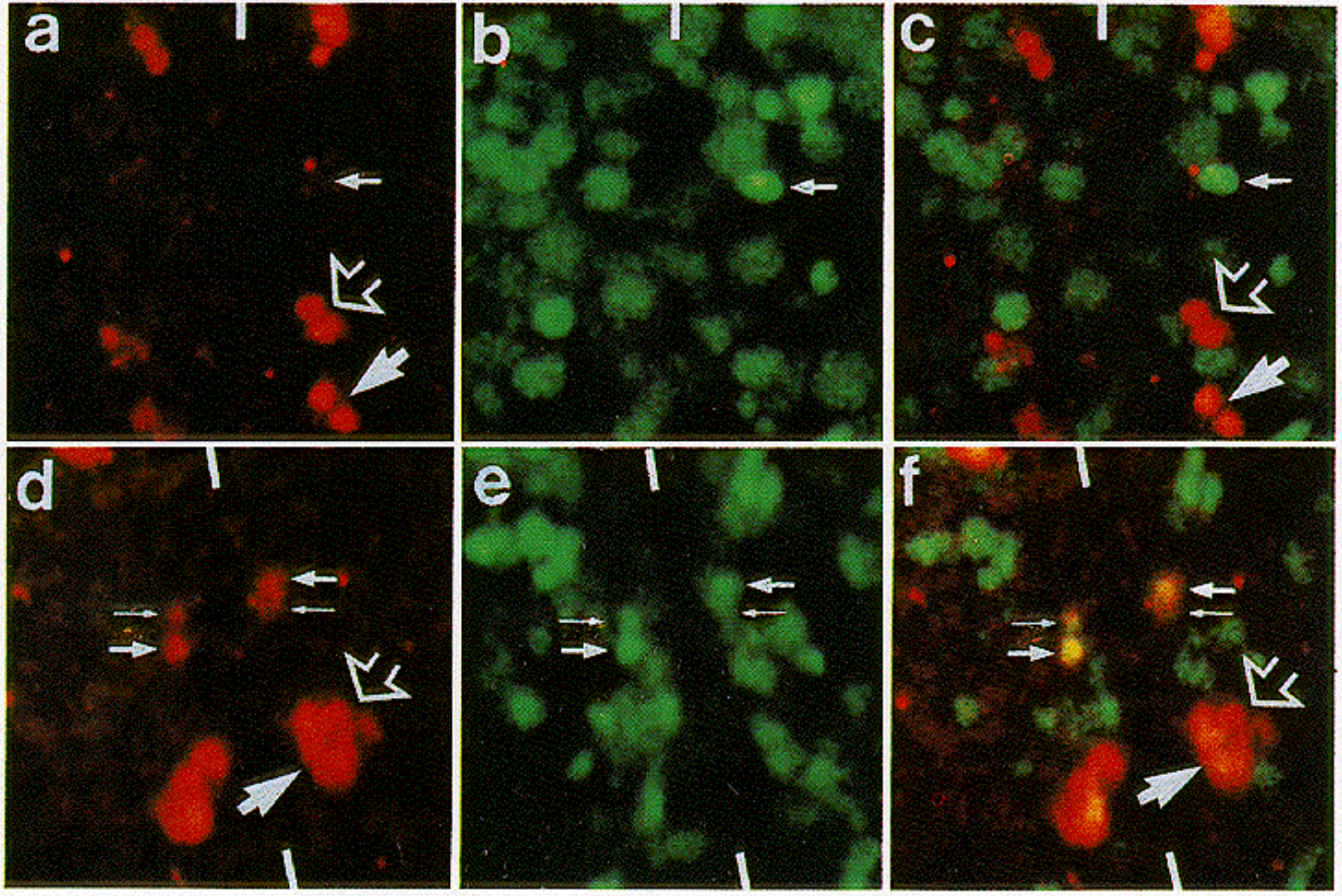
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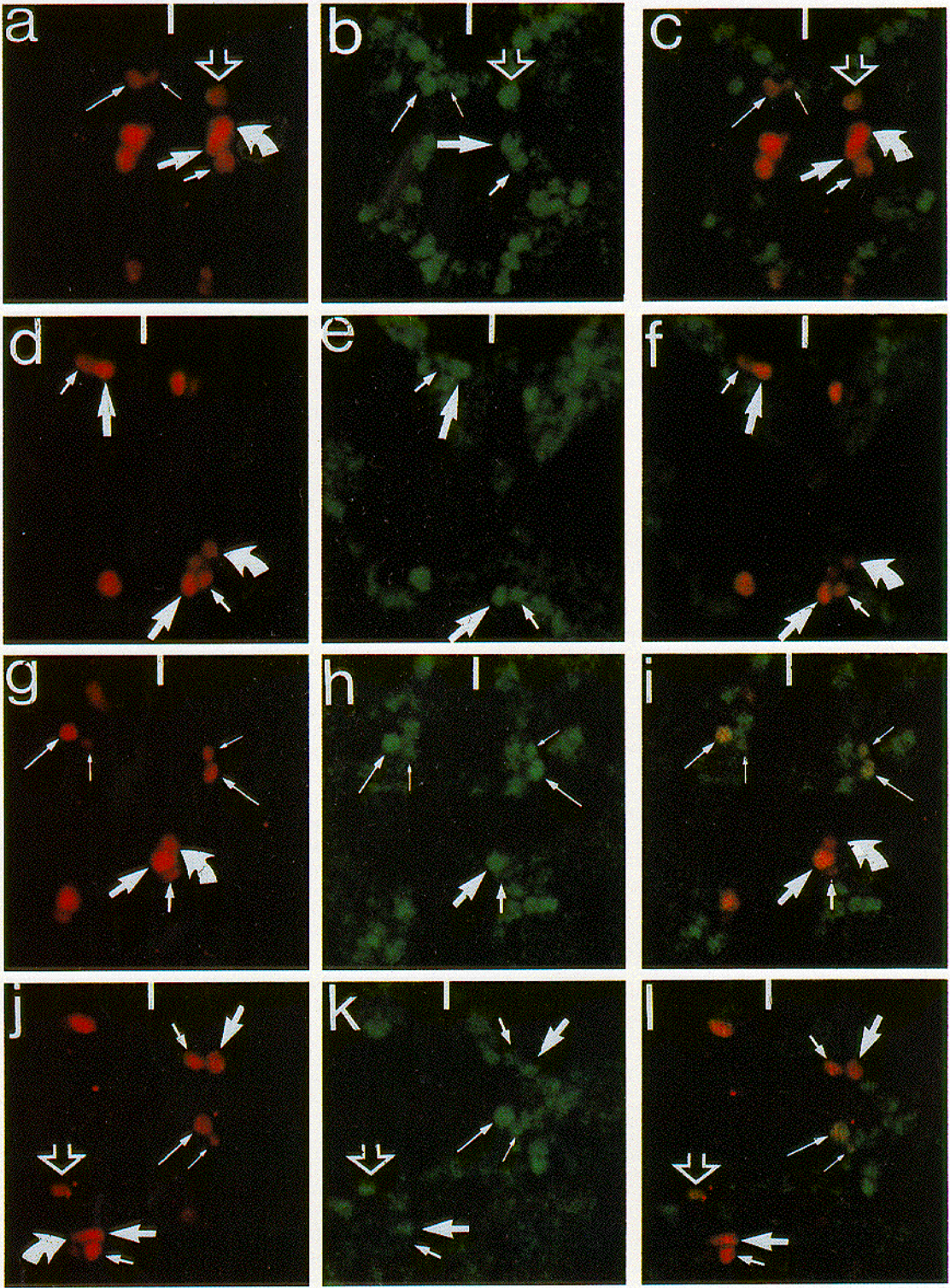
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