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# Genetic Dissection of a Complex Neurological Mutant, *polyhomeotic*, in *Drosophila*

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Null mutations at the *polyhomeotic* locus of *Drosophila* produce a complex phenotype during embryogenesis, which includes death of the ventral epidermis, misregulation of homeotic and segmentation gene expression, and global misrouting of CNS axons. It is shown here, through the use of mosaic analyses, double mutant combinations, and *in vitro* culture experiments, that all aspects of the phenotype with the exception of the axonal phenotype are cell autonomous. The changes in homeotic and segmentation gene expression in the CNS are not caused by death of the ventral epidermis, but are cell autonomous effects which most likely cause changes in neuronal cell identity. The axonal phenotype associated with *ph* mutations is also independent of epidermal cell death, but may be due to the nonautonomous effects of altered neuronal identities or to death or transformation of some as yet unidentified cell type. Despite the apparent autonomy of the *ph* mutation, mutant neurons can influence the development of adjacent wild-type neurons, presumably by depriving them of their normal fasciculation partners. 

## INTRODUCTION

The embryonic development of the insect nervous system is a complex process involving a number of discrete steps. These include the determination of the neurogenic region along the dorsal/ventral axis of the blastoderm (Anderson, 1987), the sorting out of neuroblast and epidermal lineages from within the neurogenic region (Campos-Ortega, 1988), the precise and stereotyped divisions of the neuroblasts to produce an array of identifiable neuronal progeny, and the differentiation of those progeny to produce a highly stereotyped pattern of axon fascicles and synaptic connections (Goodman *et al.*, 1984). Cell interactions are particularly important during neurogenesis in insect embryos, playing key roles during determination of neuroblasts (Doe and Goodman, 1985) and neurons (Kuwada and Goodman, 1985) and for pathfinding by neuronal growth cones. The genes which mediate these interactions in *Drosophila* are largely unknown, with the exception of the neurogenic genes, a group of genes required to inhibit ventral epidermal precursors from becoming neuronal precursors (Wright, 1970; Lehmann *et al.*, 1983; Campos-Ortega, 1988). Analysis of five of the neurogenic loci has shown that only *Enhancer of split* is cell autonomous (Technau and Campos-Ortega, 1987) and therefore likely to function as part of the response to the inhibitory signal. Another neurogenic locus, *Notch*, has been shown to be cell autonomous over large regions (Hoppe and Greenspan, 1986), but nonautonomous when small clones of mutant cells are surrounded by wild-type tissue (Technau and Campos-Ortega, 1987). This local nonautonomy suggests that *Notch* acts as an effector or signal in the inhibitory pathway, but does so with a limited diffusibility or through direct cell-to-cell contacts. The *Notch* gene encodes a transmembrane protein with extracellular domains related to epidermal growth factor (Wharton *et al.*, 1985; Kidd *et al.*, 1989), which is consistent with its proposed function.

The genes regulating other steps in neuronal development in *Drosophila* embryos are being identified by systematic screens for mutations which perturb neurogenesis in reproducible ways. One such gene is *polyhomeotic* (*ph*), a complex locus whose partial loss-of-function produces homeotic transformations in adults (Dura *et al.*, 1985). Complete loss-of-function of the *ph* locus results in an embryonic phenotype that includes death of the ventral epidermis and abnormal patterns of homeotic and segmentation gene expression (Perrimon *et al.*, 1985; Dura *et al.*, 1987; Smouse *et al.*, 1988; Dura and Ingham, 1988). In particular, normal expression of the homeotic gene *Ultrabithorax* (*Ubx*) is completely suppressed in the embryonic central nervous system (CNS) by null *ph* mutations. In contrast, the segmentation gene *even-skipped* (*eve*), which is normally expressed in only approximately 30 neurons per segment (Frasch *et al.*, 1987; Doe *et al.*, 1988), is ectopically expressed in virtually all neurons in *ph* embryos (Smouse *et al.*, 1988). There is also a dramatic and global defect in axonal guidance in the embryonic CNS. This defect results in the formation of axon bundles confined to each hemiganglion of origin ("*ph* bundles"), rather than the normal, ladder-like array of axon fascicles which com-



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prise the horizontal commissures and longitudinal connectives. To study the role of this gene during embryonic neurogenesis, we have conducted a mosaic analysis of *ph* in both neuronal and epidermal derivatives. We have addressed the question of whether cell death of the ventral epidermis is required for the nervous system phenotype or whether the alterations in the nervous system are required for epidermal cell death. Double mutant combinations have also been used to determine the requirements for the temporal and spatial specificity of cell death.

## MATERIALS AND METHODS

### *Strains*

A *ph* null mutation ($ph^{505}$) was obtained from J. M. Dura (Dura *et al.*, 1987) and is kept as $FM7c/y\ ph^{505}\ f$. $Df(1)pn^{88}$ is a deficiency of the 2D3-4 to 2E3 region which removes both *ph* and *fs(1)pecanex* (*pcx*) (Perrimon *et al.*, 1985). The unstable *ring-X* chromosome is maintained with $In(1)dl^{49},\ y\ w\ lz/Yy^{+}$ and was obtained from S. Kulkarni and J. Hall. The *armadillo* (*arm*) and *shavenbaby* (*svb*) mutations were provided by E. Wieschaus and are maintained as $FM7/y\ arm^{XK22}f$ and $FM7/w\ svb^{YP17b}$. The *dorsal* (*dl*) (Nusslein-Volhard, 1979) and *Toll* (*Tl*) (Anderson *et al.*, 1986) stocks were kept as $dl^{1}$, *cn bw sp/CyO* and $Tl^{9Q}$ *rucuca/T(1;3)OR60/TM3, Sb Ser*, respectively. The *string* (*stg*) (Jurgens *et al.*, 1984) mutation was obtained from the Bowling Green Stock Center and is kept as $stg^{7M53}$ *rucuca/TM3, Sb Ser*. The *engrailed/lacZ* stock was provided by C. Hama and T. Kornberg.

Flies were raised on standard *Drosophila* media at 25°C. Descriptions of balancers and mutations if not otherwise described in the text can be found in Lindsley and Grell (1968).

### *Mosaic Analyses*

Larval and embryonic gynandromorphs were generated by the loss of an unstable *ring-X* chromosome. Larvae were derived from a cross between $FM7c/y\ ph^{505}\ w\ svb^{YP17b}$ females and *ring-X* males for analysis of the cuticular pattern, while embryos used for analysis of CNS mosaics were produced from a cross between $FM7c/\ y\ ph^{505}\ f$ females and *ring-X* males.

Mitotic recombination during embryonic development was induced as described by Wieschaus and Riggleman (1987). Virgin females heterozygous for $arm^{XK22}$, or $arm^{XK22}\ ph^{505}$ were mated to wild-type *Oregon R* (*OreR P2*) males. Eggs were collected hourly and aged at 25°C for approximately 3.5 hr. After X irradiation of 500 rad, (Torrex 120D X-ray machine; 100 kV, 5 mA, 3 mm aluminum filter), embryos were allowed to develop for 36 hr at 22°C before mounting. All larvae and unhatched eggs were dechorionated in 50% Chlorox bleach, heated in glycerol/acetic acid, and mounted in Hoyers mountant as described by van der Meer (1977). Cuticle preparations were scored for gross phenotype and incidence of ectopic denticles using phase-contrast and Nomarski optics at 200 to 400×.

### *Single Embryo Culture*

Single embryo culture was performed as a modification of published techniques (Furst and Mahowald, 1985). Single, 4- to 6-hr embryos from a cross of $FM7c/\ y\ ph^{505}\ f$ females to $FM7c/Y$ males were manually dissociated with sterile, pulled glass pipets; the resulting cell suspensions were plated on glass coverslips and maintained in conditioned Schneider's media supplemented with 15% fetal calf serum at 25°C for 12–24 hr. The cell density of these cultures was too low to support normal cell division and differentiation; it was therefore necessary to grow the cells in media that had been conditioned by high density cell cultures from wild-type embryos and subsequently filtered with 0.22-$\mu$m filters. Cultures were fixed for 10 min in 0.1 *M* Pipes (pH 6.9), 1 m*M* EGTA, 2 m*M* $MgSO_4$, 7% formaldehyde and stained with antibodies as below.

### *Immunohistochemistry*

Embryos were prepared for immunohistochemistry using minor modifications of published procedures (Mitchison and Sedat, 1983); histochemical localization of horseradish peroxidase was performed as described (Ghysen *et al.*, 1986).

Embryos were dechorionated in 50% Chlorox bleach, fixed for 10 min in PBS-buffered 4% paraformaldehyde/heptane, and devitellinized with absolute methanol. All steps were performed at room temperature. Following fixation, embryos and cell cultures were washed in PBS + 0.1% Triton X-100 (PT) and incubated overnight at 4°C with the appropriate primary antisera. Primary antibodies included mouse anti-*Ubx* antibody (dilution, 1:1; obtained from R. White), mouse anti-BP102 (dilution, 1:1; this is a monoclonal antibody from A. Bieber and C. Goodman which stains CNS axons), and rabbit anti-*eve* (dilution, 1:50; obtained from M. Frasch). Embryos and cultures were then washed in PT for 2-4 hr at room temperature and incubated overnight at 4°C with biotinylated horse anti-mouse IgG (dilution, 1:500; Vector), biotinylated goat anti-rabbit IgG (dilution, 1:500; Vector), or a mixture of fluorescein-conjugated goat anti-mouse IgG (dilution, 1:500; Cappel) and rhodamine-conjugated goat anti-HRP (dilution, 1:100; Cappel). Embryos and cultures were washed in PT for 2-4 hr at room temperature.

Visualization of horseradish peroxidase was performed using a Vectastain ABC kit. Peroxidase-labeled preparations were dehydrated in ethanol, cleared in methyl salicylate, and viewed using a Zeiss Axiophot microscope with Nomarski optics. Fluorescently labeled preparations were cleared in 90% glycerol + 4% *n*-propyl gallate.

## RESULTS

### *The Epidermal Cell Death Caused by polyhomeotic Is Cell Autonomous*

The cell death observed in *ph* embryos has a high degree of temporal and spatial specificity (Dura *et al.*, 1987; Smouse *et al.*, 1988). Death of the ventral epidermis is not apparent until 9–10 hr of development, or approximately 4 hr after the neuroblasts have separated from the neuroepithelium and at a time when axon pathways are being formed in the CNS. Cell death is confined to epidermal cells which are derived from the ventral neurogenic region of the blastoderm fate map; that is, all epidermal cells ventral to the tracheal placodes (Lohs-Shardin *et al.*, 1979; Campos-Ortega and Hartenstein, 1985). A very precise boundary forms between the surviving lateral epidermis and the missing ventral epidermis (see Fig. 1 in Smouse *et al.*, 1988), suggesting a certain spatial autonomy to the cell death. Epidermis associated with other nervous system derivatives, such as the brain lobes and peripheral neurons, survives and secretes cuticle.

Does the ventral epidermis die in mutant embryos because of the loss of *ph* function in those cells? Or is it loss of *ph* in some other tissue, such as the CNS or mesoderm, which then leads to the loss of a signal normally required for ventral epidermal viability? These questions address the cell autonomy of the mutant phenotype, and a functional assay of cell autonomy is to introduce mutant cells into a wild-type environment. If the mutant cells are rescued by the surrounding wild-type cells, then the mutation is considered nonautonomous. If, however, the mutant phenotype persists despite the presence of wild-type tissue, then the mutation is considered autonomous. A classic way to introduce mutant cells in a wild-type background is to use the unstable *ring-X* chromosome, which is lost early in development during the first mitotic divisions of the zygotic nuclei. Loss of the *ring-X* chromosome causes embryos to be mosaic for sex, i.e., some regions of the embryo will remain *rod-X/ring-X* and be female, while those derived from cells that have lost the *ring-X* will be *rod-X/O* and male (Zalokar *et al.*, 1980). If the *rod-X* chromosome carries an embryonic lethal mutation, such as *ph*, then the mosaic embryos or gynandromorphs will test the global autonomy of that mutation (Gergen and Wieschaus, 1986; Hoppe and Greenspan, 1986), since large patches of genotypically mutant tissue (*rod-X/O*) will be juxtaposed with genotypically wild-type tissue (*rod-X/ring-X*).

To detect *ph* mutant cells in mosaic embryos, a second mutation, which produces a scorable, cell-autonomous phenotype, must be linked to *ph*. *shavenbaby* (*svb;* 4E) (Wieschaus *et al.*, 1984; Gergen and Wieschaus, 1986) is an *X*-linked, cell-autonomous mutation which reduces the size and number of all larval denticles. Females heterozygous for a chromosome carrying both the *ph* and *svb* mutations (*FM7c/y* $ph^{505}$ *w* $svb^{YP17b}$) were crossed to males carrying the unstable *ring-X* chromosome and cuticles of the resulting larvae were examined. If the *ph* mutant phenotype is expressed nonautonomously, then some mutant cells will be rescued from death by neighboring wild-type cells. The rescued mutant epidermis will be mutant for *svb* and will secrete denticles that are morphologically distinguishable from wild-type. If, on the other hand, *ph* behaves autonomously, then all mutant cells will die and no *svb* denticles will be observed. Twenty-one mosaic embryos were analyzed and none had clones of *svb* denticles. Figures 1C and 1D show an example of such a gynandromorph. The mosaic embryo has a region of ventral epidermis that is viable and has wild-type denticles and a large patch of mutant tissue that has died prior to secreting cuticle. A small patch of naked cuticle can be seen in the mutant patch, but it does not contain *svb* denticles; similar patches of naked cuticle are also occasionally found in the ventral cuticle of *ph* null embryos (Fig. 1A). These results indicate that the epidermal cell death caused by the *ph* mutation is autonomous in large clones of cells.

The *ring-X* chromosome is lost early in embryogenesis and therefore results in large patches of mutant and wild-type tissue (Zalokar *et al.*, 1980). If nonautonomous interactions are mediated by cell–cell contacts, rather than by diffusible substances, then mutant cells may not be rescued when they are present in large clones. The autonomy of the *ph* mutation was therefore tested by a second approach designed to produce small clones of mutant cells surrounded by wild-type cells. X-ray irradiation of embryos at the cellular blastoderm stage induces mitotic recombination between homologous chromosomes; if one of the homologs carries the *ph* mutation, then recombination followed by independent assortment of the chromosomes will result in clones of homozygous mutant cells adjacent to homozygous wild-type cells. The small size of *svb* denticles makes it difficult to score in X-ray-induced clones; however, the *X*-linked segment polarity mutation, *armadillo* (*arm;* 2B17) causes the cell-autonomous formation of ectopic denticles with normal size and reversed polarity in the

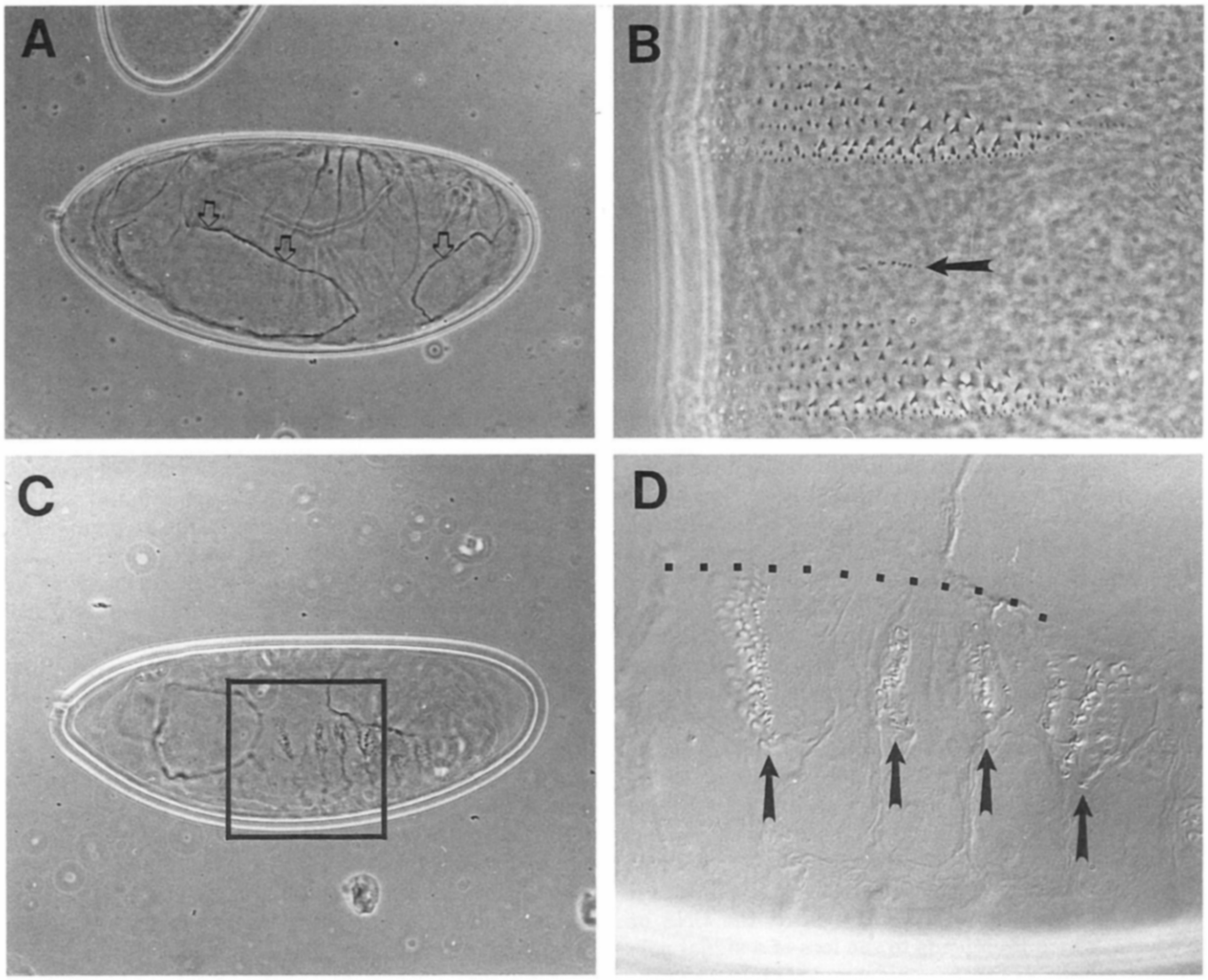



FIG. 1. Mosaic analysis of *polyhomeotic* epidermis. (A) Side view of an embryo hemizygous for $ph^{505}$. Most of the ventral cuticle is missing and there are no denticle bands on the small region of cuticle which has been secreted by surviving epidermal cells. Arrows indicate the border between the missing ventral cuticle and the naked lateral cuticle. Anterior is to the left and ventral is to the bottom. (B) An example of an epidermal clone (indicated by arrow) induced in an embryo heterozygous for $arm^{XK22}$ $ph^{505}$. This clone contains nine denticles pointing posteriorly. Anterior is up. (C) A mosaic embryo of the genotype $y$ $ph^{505}$ $svb^{YP17b}$/*ring-X*. Note the border between wild-type and mutant territories in which there is no cuticle; as in (A), there is a region of surviving, naked cuticle which is presumably mutant. (D) Normarski enlargement of the area boxed in (C). Note the wild-type territory with denticle belt (indicated by arrows). The border between wild-type and presumed mutant territory is delineated by dots.

normally naked, posterior regions of all segments (Wieschaus *et al.*, 1984; Wieschaus and Riggleman, 1987). To analyze the cell autonomy of *ph* in small clones, *arm* and *ph* mutations were recombined onto the same chromosome, mitotic clones were induced at the blastoderm stage, and the occurrence of *arm* denticles was scored in these larvae. If the epidermal cell lethality caused by the *ph* mutation is indeed cell autonomous, then no *arm* clones should be detected. Two sets of genotypes were irradiated. As a control, *FM7/y* $arm^{XK22}$ *f* females were crossed to wild-type males, and the frequency of clones among larvae heterozygous for $arm^{XK22}$ alone was found to be 28% (22 clones) and the average size of epidermal clones induced by mitotic recombination was 2.9 denticles. These data are consistent with the frequency and size of *arm* clones observed by Wieschaus and Riggleman (1987). No significant differences were observed when *ph* was linked in *cis* with *arm*. The frequency of clones among progeny heterozygous for both $arm^{XK22}$ and $ph^{505}$ (*FM7/y* $arm^{XK22}$ $ph^{505}$ *f*

females crossed to wild-type males) was 24% (25 clones) and the average size was 2.6 denticles (Fig. 1B). Thus, small clones of *ph* mutant cells survive, divide at least once or twice, and secrete cuticle.

The two different methods used to induce mutant clones, *ring-X* loss and X-ray irradiation, give different results that appear to be contradictory. However, the apparent rescue of small blastoderm clones does not necessarily indicate that *ph* is nonautonomous at the single cell level. Since only one or two cell divisions occur in the epidermis (Szabad *et al.*, 1979; Campos-Ortega and Hartenstein, 1985; Wieschaus and Riggleman, 1987) following irradiation, it is quite likely that there is perdurance of wild-type *ph* gene product, synthesized prior to clone induction, which prevents cell death. Only clones destined to divide once or twice would have sufficient gene product to prevent cell death, while larger mutant clones, such as those induced by loss of the *ring-X*, would presumably dilute, during mitosis, perduring stores of gene product to levels unable to support cell viability. Indeed, limited survivability of *ph* mutant cells for several divisions was also observed when *ph* mutant clones were induced during imaginal disc development (Santamaria *et al.*, 1989). These results, taken together, indicate that epidermal cell death is cell autonomous and that perdurance of wild-type *ph* function can rescue mutant cells from death for up to two cell divisions.

### *Interactions between polyhomeotic and Mutations Affecting the Dorsal/Ventral Axis*

The cell death caused by loss of *ph* function is precisely confined to the ventral epidermis of the embryo. Is this specificity due to the position or the identity of the affected cells? To address this question, double mutant combinations were made between *ph* and mutations which alter the dorsal/ventral identities of epidermal cells (see review by Anderson, 1987). Mutations at the *dorsal* (*dl*) locus (Nusslein-Volhard, 1979) cause dorsalization of the lateral and ventral cells of the blastoderm fate map. This results in tube-shaped embryos with no ventral cuticle and little or no mesoderm and CNS (Nusslein-Volhard, 1979; Campos-Ortega, 1983). Dominant, gain-of-function mutations at the *Toll* ($Tl^D$) locus produce the opposite phenotype, causing ventralization of the dorsal cells of the fate map (Anderson *et al.*, 1986). This results in embryos with ventral denticle bands on both dorsal and ventral surfaces; in some cases, these denticle bands circle the embryo. Internally, it results in a greatly expanded and diffuse CNS which extends axons chaotically throughout the embryo (Figs. 3D, E) (see also Campos-Ortega, 1983). If *ph* responds to the determinants of the dorsal/ventral axis, then dorsalization should rescue the presumptive ventral epidermis from cell death, and ventralization should cause death of the presumptive dorsal epidermis. If, on the other hand, cell death caused by *ph* mutations is dependent on position, then dorsalized and ventralized embryos should still exhibit cell death confined to the presumptive ventral epidermis.

Embryos doubly mutant for *ph* and either *dl* or $Tl^D$ mutations were collected and stained with antibody probes to analyze either the cell death phenotype or the CNS phenotypes which are normally associated with *ph*. Antibodies against *Ubx* or *eve* proteins were particularly useful, since they allowed embryos mutant for *dl* or $Tl^D$ to be distinguished from those embryos doubly mutant for a dorsal/ventral gene and *ph*. Loss of *ph* function causes the repression of *Ubx* and the derepression of *eve* in embryonic neurons (Smouse *et al.*, 1988). Antibodies against HRP, which label all neuronal cell bodies and axons (Jan and Jan, 1982), were used to analyze CNS phenotypes and, in some cases, anti-*Ubx* and anti-HRP were used to doubly label embryos.

Females homozygous for *dl* and heterozygous for $ph^{505}$ were crossed to wild-type males; all of the progeny from this cross were $dl^-$ and one-quarter were also mutant for *ph*. A small ring of $eve^+$ cells which girdle part of the posterior midgut was present in *ph* and *ph;dl* embryos, but was not present in wild-type or *dl* embryos (compare Figs. 2A and 2B). These cells were useful in distinguishing *ph;dl* from *dl* embryos. There was an intact tube of epidermis in *ph;dl* mutant embryos (Fig. 2B) that was essentially identical to the epidermis found in *dl* embryos (Fig. 2A). Thus, dorsalization of the presumptive ventral epidermis rescued it from cell death. The only evidence of cell death was at the anterior and posterior termini of the double mutant embryos. Small clusters of neuronal tissue at the anterior ends of the *ph;dl* embryos were observed occasionally; these cells expressed *eve* and the anti-HRP antigen, but did not express *Ubx*. Because of their infrequent occurrence and relatively small size, it was not possible to discern a distinct axonal pattern within these neuronal clusters.

The opposite phenotype was observed in *ph*; $Tl^D$ embryos, where ventralization of the epidermis resulted in death of virtually all epidermal cells. Females heterozygous for $Tl^D$ and $ph^{505}$ were crossed to males carrying an *engrailed/lacZ* construct (T. Kornberg, personal communication) which directs the expression of β-galactosidase in *en*-specific stripes in the epidermis. Anti-β-galactosidase antibodies demonstrated that the *en*-specific stripes were expressed in the epidermis of embryos mutant for $Tl^D$, although the morphology of the stripes was abnormal (Fig. 2C). In embryos mutant for both *ph* and $Tl^D$ (Fig. 2D), the *en* stripes were diffuse and indistinct, as in *ph* embryos (Smouse *et al.*, 1988). More im-

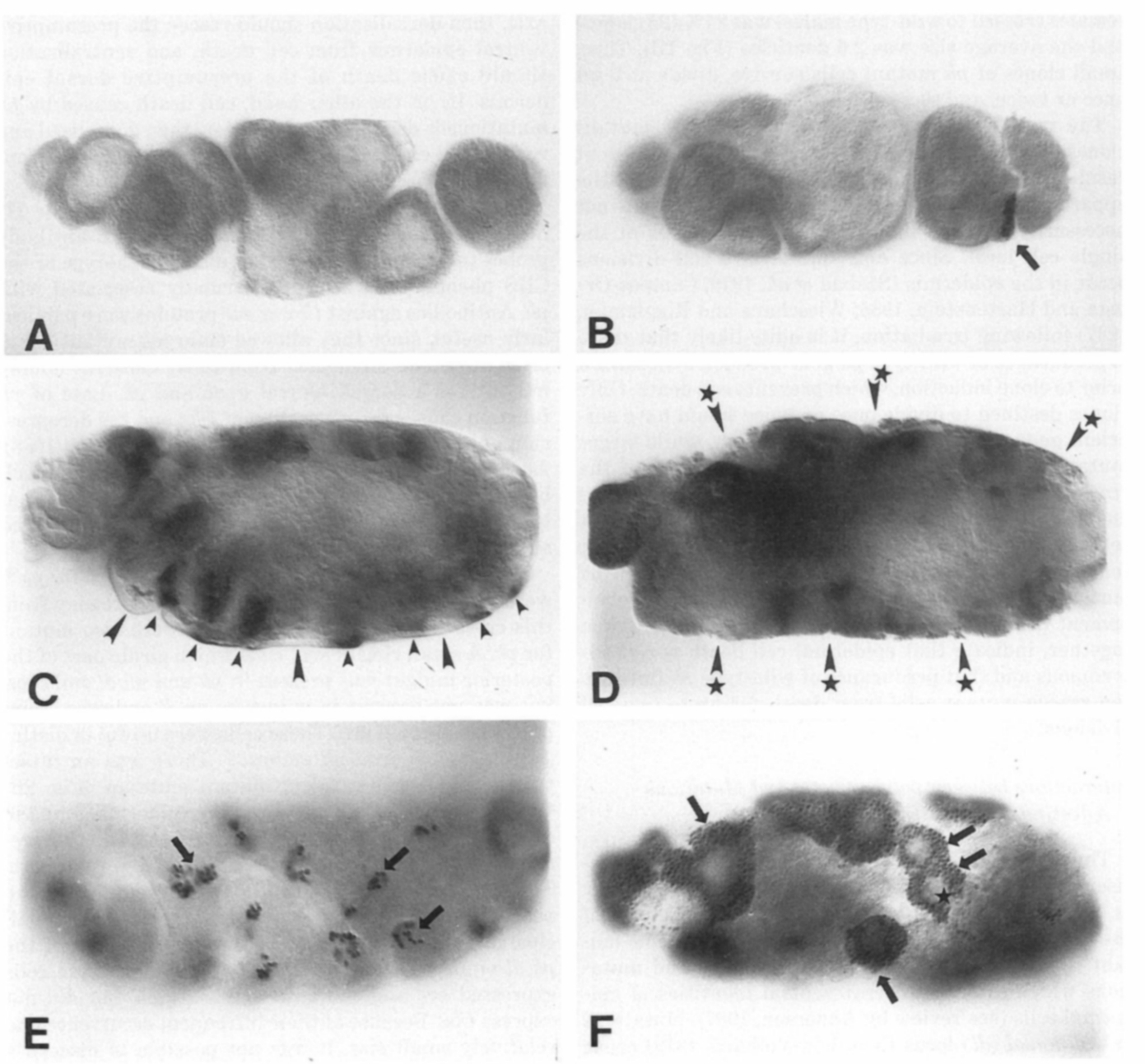



FIG. 2. Phenotype of *polyhomeotic;dorsal* and *polyhomeotic;Toll* double mutant embryos. In all panels, anterior is to the left. (A) A *dl* embryo at 12-13 hr stained with anti-*eve;* the embryo resembles a tube of epidermis. There are no $eve^+$ cells present. (B) A *ph;dl* embryo showing the small ring of $eve^+$ cells in the posterior midgut (arrow). While it is difficult to distinguish the presumptive "dorsal" surface from the "ventral," there are no obvious signs of cell death in any region of the epidermis. (C) β-Galactosidase expression in a $Tl^D$ mutant embryo containing an *engrailed/lacZ* construct. The *en* stripes are present (small arrowheads) but not quite normal because of the altered dorsal/ventral polarity of the embryo. The morphology of the head was used to orient the embryo; in (C-F), the presumptive dorsal surface is up and the presumptive ventral surface is down. The presumptive ventral epidermis is viable and visible in Nomarski optics (large arrowheads). (D) β-Galactosidase expression in a $ph;Tl^D$ mutant embryo containing the *en/lacZ* construct. The *en* pattern is much weaker, more diffuse, and no longer expressed in obvious stripes, indicating that this embryo is mutant for *ph*. There are dying cells on both presumptive dorsal and ventral surfaces of the embryo (starred arrowheads). (E) A $Tl^D$ embryo stained with anti-*eve*. Small clusters of neurons expressing *eve* are found throughout the embryo (arrows). (F) A $ph;Tl^D$ embryo stained with anti-*eve*. Note the ectopic expression of *eve* in all neurons (arrows), which seem to have organized into small, circular clusters of cells. The star indicates an unstained axon bundle in the center of a cluster. As in (D), there is necrotic epidermis on both "dorsal" and "ventral" surfaces.

portantly, the epidermis on both the presumptive ventral and dorsal surfaces was dead or dying. The death of the epidermal cells gave the embryos a ragged appearance, in contrast to the normally smooth contours of wild-type or $Tl^D$ embryos. The CNS neurons in these double mutant embryos did not express *Ubx*, but did express *eve* ectopically (Fig. 2F). Furthermore, when *ph*;$Tl^D$ embryos were doubly labeled with anti-*Ubx* and anti-HRP antibodies, it was found that the doubly mutant neurons organized into small, ganglion-like clusters of cells. The axons from a single cluster formed an axon bundle (Figs. 3F and 3G) similar to the axon bundles characteristic of *ph* embryos (Fig. 3C). These bundles are quite striking, considering the dispersed nature and random location of the neuronal clusters which are the result of the $Tl^D$ mutation and are in sharp contrast to the diffuse and disorganized arrays of axons observed in embryos mutant only for $Tl^D$ (Figs. 3D and 3E).

These observations indicate that the epidermal cell death caused by *ph* mutations depends on the dorsal/ventral identities of the affected cells and is not simply the result of the position these cells occupy. Furthermore, the ectopic neurons that develop in ventralized embryos exhibit appropriate *ph* phenotypes—they fail to express *Ubx*, they overexpress *eve*, and their axons even form "*ph* bundles." This argues that these phenotypes are dependent on the genotype of the affected neurons and independent of their position in the embryo.

### *Inhibition of Cell Division Rescues Cell Death*

We have proposed that *ph* mutations cause cell death in an autonomous way that depends on the dorsal/ventral identities of the mutant cells and on the number of divisions they perform. If the latter hypothesis is true, then perturbing the number of cell divisions should have an affect on the mutant phenotype. A gene on the third chromosome, *string* (*stg*) (Jurgens *et al.*, 1984; Edgar and O'Farrell, 1989), is required for continuation of mitosis after the 13th nuclear division. Despite the absence of further cell division, embryos mutant for *stg* undergo many of the morphogenetic movements and differentiation steps characteristic of normal embryos. For instance, neuroblasts enlarge and delaminate from the neuroectoderm in a nearly normal pattern and with a nearly normal time course in *stg* embryos. Similarly, the epidermal cell precursors secrete cuticle at the appropriate time, although this cuticle is poorly differentiated. Embryos doubly mutant for *stg* and *ph* were compared to embryos mutant for *stg* or *ph* alone to assess the effect of mitotic inhibition on the *ph* phenotypes. Mutant embryos were again examined for the expression of *Ubx* and *eve* in the CNS and for viability of the ventral epidermis. *stg* embryos had a *Ubx* pattern that was grossly similar to that of wild-type embryos (data not shown) and an *eve* pattern that was characterized by a small number of positively stained neuroblasts (2–4) per segment (Fig. 4B). In contrast, *ph;stg* mutant embryos had no *Ubx* expression and ectopic *eve* expression in virtually all neuroblasts of the brain and ventral nerve cord (Fig. 4D). Furthermore, the ventral epidermis in these double mutant embryos remained viable (Fig. 4D) and was able to secrete cuticle which was similar to the cuticle secreted by *stg* embryos (data not shown). Thus, the absence of cell division rescues *ph* ventral epidermal cells from the expected cell death, but does not rescue the changes in *Ubx* and *eve* expression in mutant neural cells.

### *Interaction between polyhomeotic and a Neurogenic Mutation*

Mutations in neurogenic loci cause an overproliferation of neurons by committing presumptive epidermal cells to become neuroblasts (Wright, 1970; Lehmann *et al.*, 1983). Embryos were made mutant for both a neurogenic gene and *ph*, to determine if the extra neurons produced by a neurogenic mutation would form bundles in a *ph* embryo or if the neurogenic mutation, presumably epistatic to *ph*, would produce a phenotype characteristic of the neurogenic mutant alone. The deficiency, $Df(1)pn^{88}$, removes both *ph* and a paternally rescuable, maternal effect neurogenic locus, *fs(1)pecanex* (*pcx*; 2E2-3) (Perrimon *et al.*, 1984; LaBonne and Mahowald, 1985). Females heterozygous for $Df(1)pn^{88}$ were crossed to males hemizygous for *pcx*. The resulting female progeny of the genotype $Df(1)pn^{88}/pcx$, when mated to *pcx*/*Y* males, produced two phenotypic classes of lethal progeny: the first class consisted of embryos hemizygous or homozygous for *pcx* or heterozygous for $Df(1)pn^{88}$ and *pcx*. All embryos of this class should have a neurogenic phenotype associated with *pcx*, since they were derived from *pcx*-deficient mothers, and all should carry at least one wild-type copy of the *ph* locus. The second class of mutant embryos consisted of male embryos of the genotype $Df(1)pn^{88}/Y$, and these, lacking both maternal and zygotic *pcx* gene expression, should be neurogenic and, lacking *ph* gene activity, should be *ph* as well. The absence of *Ubx* expression in *ph* mutants was again exploited to distinguish between these classes of mutant embryos.

The extra neurons in *pcx* mutants expressed *Ubx* in a pattern similar to that of wild-type neurons (Fig. 5a). The extra neurons also attempted to form commissures and connectives (Fig. 5b), although these were abnormal and disorganized. Many axons wandered randomly within the CNS and some strayed into the periphery.

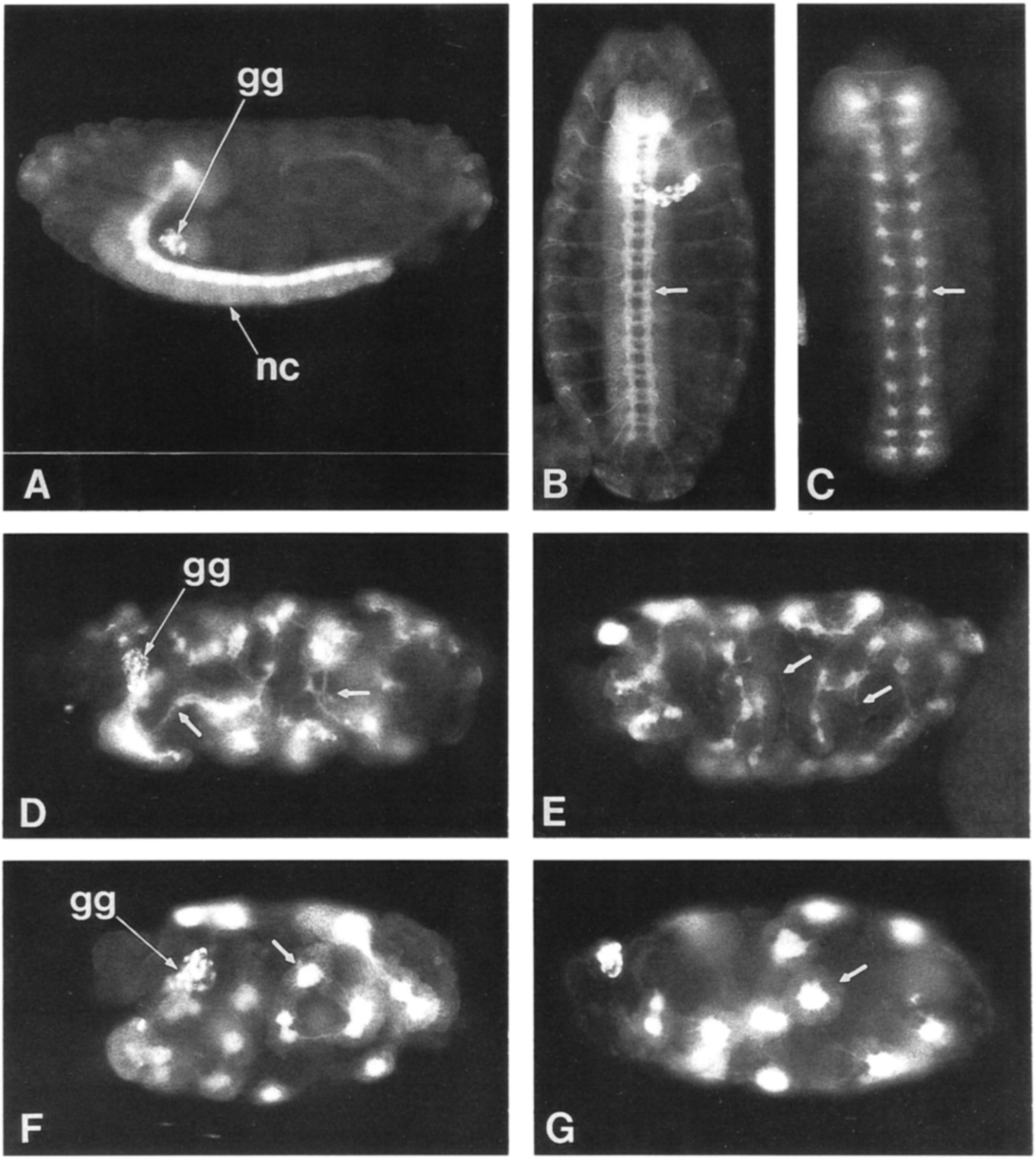



FIG. 3. CNS phenotype of *Toll* and *polyhomeotic;Toll* mutant embryos. All panels show embryos stained with anti-HRP. (A) Wild-type embryo shown in side view with anterior to the left and dorsal up. The ventral nerve cord (nc) and the garland gland (gg) are intensely labeled. (B) Wild-type embryo in ventral view, showing the ladder-like array of axons. The arrow points to a typical pair of commissures within one segment. Anterior is up. (C) A *ph* embryo in ventral view, showing the axon bundles which have replaced the normal ladder-like array. The arrow points to the "*ph* bundle" within a hemiganglion. (D, E) $Tl^D$ embryos have a diffuse distribution of neurons and extensive arrays of axons (arrows). Note that axons can be seen growing throughout the embryos. (F, G) *ph*;$Tl^D$ embryos have bundles of axons characteristic of *ph* embryos (arrows). The embryos in (D–G) were also labeled with anti-*Ubx* to independently confirm their genotypes (data not shown).

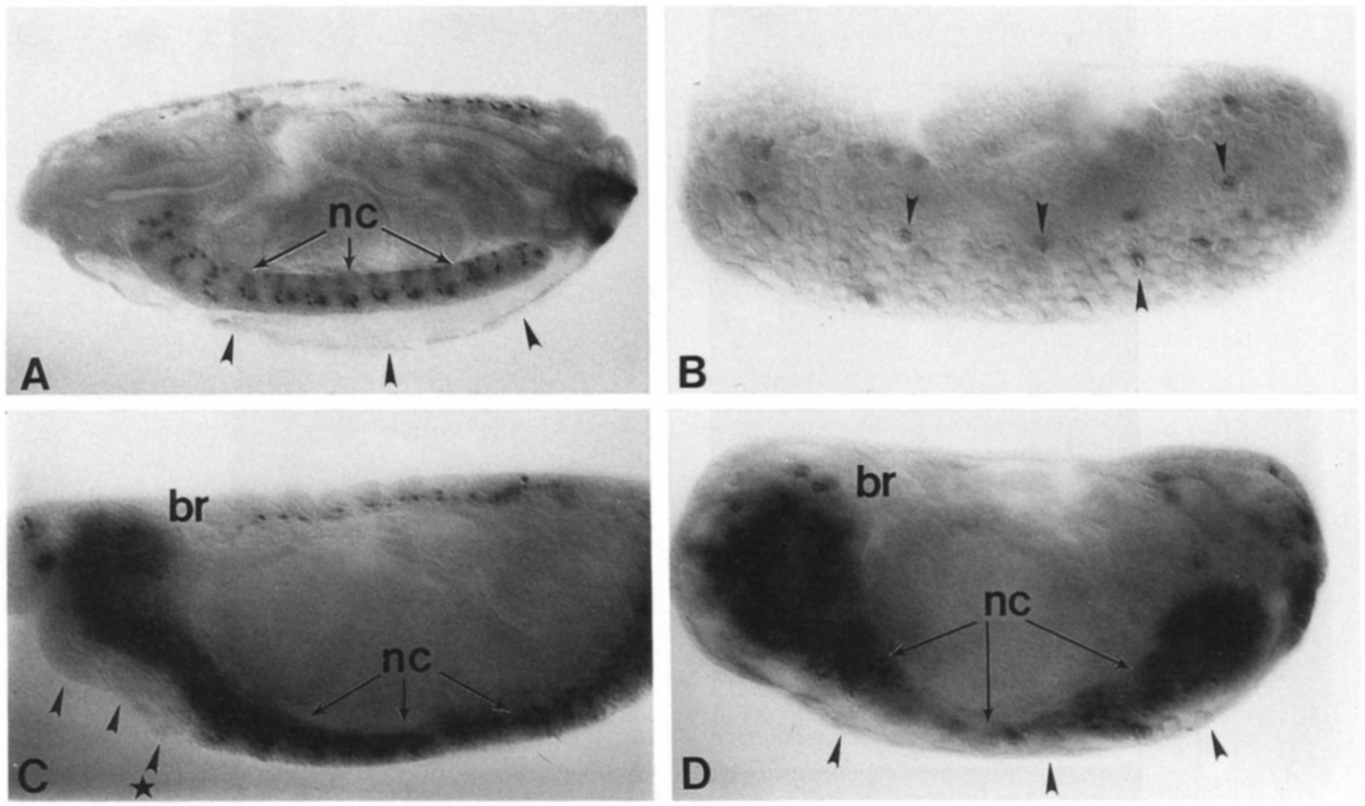



FIG. 4. Phenotype of *polyhomeotic;string* double mutant embryos. All panels show side views of embryos stained with anti-*eve;* anterior is to the left. (A) Wild-type embryo at 12–13 hr of development. The arrows indicate the ventral nerve cord (nc) in which a small number of neuronal nuclei stain positively. The arrowheads point to the ventral epidermis. (B) A *stg* embryo showing the small numbers of $eve^+$ neuroblasts (arrowheads). This embryo is slightly younger than the others shown and is photographed in a superficial lateral view to demonstrate the $eve^+$ neuroblasts. (C) A *ph* embryo showing ectopic *eve* expression throughout the ventral nerve cord (nc) and brain (br). The arrowheads point to the surviving ventral epidermis, which consists of a small patch of cells at the anterior end. The starred arrowhead indicates a small cluster of necrotic cells. (D) A *ph;stg* double mutant embryo. The ventral nerve cord (nc) and brain (br) consist of undivided neuroblasts which express *eve* ectopically as do the neurons in embryos mutant only for *ph.* The arrowheads point to the ventral epidermis, which is still viable and intact.

The extra neurons in embryos lacking both *pcx* and *ph* failed to express *Ubx* (Fig. 5c) and, remarkably, formed bundles characteristic of *ph* embryos (Fig. 5d). The bundles appeared to contain all the axons originating in a given hemiganglion, as no stray axons were observed. The bundles were also larger than those formed in embryos lacking *ph* alone, as would be expected if they contained the axons from more than the normal number of neurons. Thus, transformation of epidermal cell precursors to neuroblasts rescues them from cell death, again indicating that cell death is specific to cell fate rather than position. Since the neuronal progeny of these neuroblasts continue to form the axon bundles characteristic of *ph,* it would also seem that epidermal cell death is not required for the formation of those bundles.

### *Mosaic Analysis of the Polyhomeotic Nervous System*

The nervous system phenotype caused by the *ph* mutation is an unusual perturbation of the very regular and orthogonal array of axons seen in the wild-type embryo (Smouse *et al.*, 1988). In wild-type embryos, the axons from the CNS neurons organize into a ladder-like neuropil composed of a pair of horizontal commissures per segment or ganglion and a pair of longitudinal connectives which carry axons between ganglia (see Fig. 3B). In *ph* embryos the axons of a single hemiganglion fasciculate together at the center of that hemiganglion, to form a large bundle, and these axons rarely escape to the contralateral hemiganglion or to adjacent segments (see Fig. 3C). Gynandromorphs mosaic for *ph* were generated as described for the epidermis and were analyzed for the cell autonomy of this CNS phenotype. To identify clones of mutant tissue, *y* $ph^{505}f$*/ring-X* embryos were doubly stained with either anti-*Ubx* and anti-HRP or anti-*eve* and anti-BP 102, a mouse monoclonal antibody which labels all CNS axons. Hemizygous mutant clones in the CNS were identified by the absence of *Ubx* expression or by the overexpression of *eve,* and the axonal phenotypes of the clones and surrounding wild-type neurons were examined. Clones of different sizes

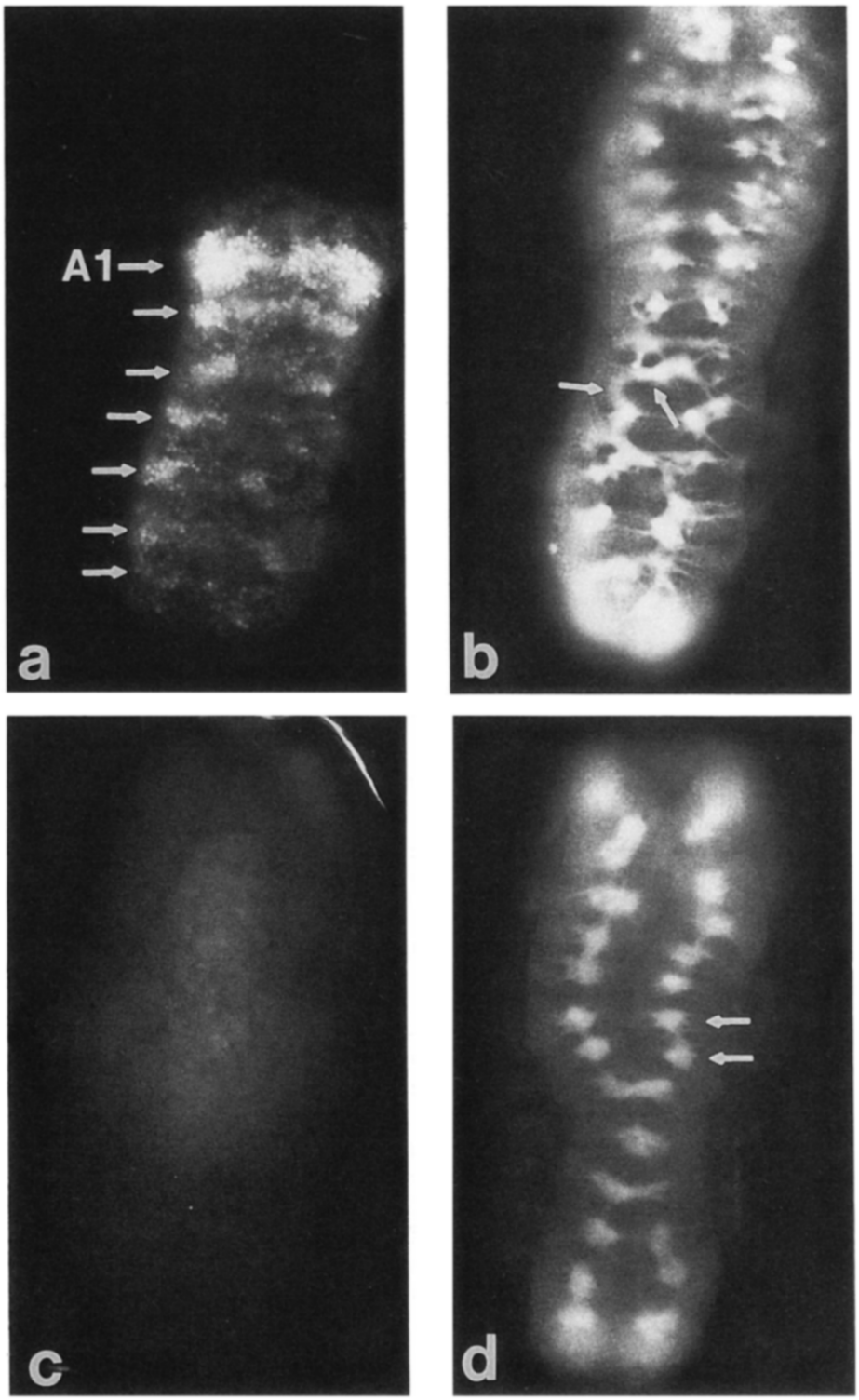



FIG. 5. Interaction between *polyhomeotic* and a neurogenic mutation. In all panels, anterior is up. (a, b) A *pcx* embryo derived from a $Df(1)pn^{88}/pcx$ mother and stained with anti-*Ubx* (a) and anti-HRP (b). The arrows indicate different segments of the nerve cord in which neurons express *Ubx* (A1–A7). In (b) the arrows point to axons growing within or between segments. (c, d) A $Df(1)pn^{88}/Y$ embryo derived from a $Df(1)pn^{88}/pcx$ mother, stained with anti-*Ubx* (c) and anti-HRP (d). There are no neuronal nuclei which express *Ubx;* the faint fluorescence in (c) is due to autofluorescence of the yolk. Note the organized array of "*ph* bundles" in (d). The two bundles of a segment are generally further apart than in an embryo mutant for *ph* alone, which is expected if the bundles are separated by extra neurons. The reproducible exception to this is that the bundles of several abdominal segments fuse along the midline; this fusion is sometimes seen in embryos mutant for *pcx*, and is possibly due to a defect occurring during the extended germ band stage.

and with variable boundaries were observed in the CNS. Clones which spanned a segment or more had an axonal phenotype like that of *ph*—the neurons belonging to mutant segments formed "*ph* bundles" rather than the connectives and commissures of the wild-type CNS (Figs. 6D1 and 6D2). The ventral epidermis associated with such clones was usually missing. The patterns of *Ubx* or *eve* expression in neurons surrounding the clones, and the associated ventral epidermis, appeared normal, but the axonal phenotype was dependent on their position relative to the clone. Wild-type neurons immediately adjacent to the clone often had abnormal axon patterns, but were clearly different from the *ph* pattern. Typically, they had incomplete connectives and commissures which were thinner than normal. Neurons in segments not immediately adjacent to *ph* clones had normal axon morphology. Small clones comprising less than a hemisegment in size were also detected on the basis of ectopic *eve* expression (Figs. 6B1 and 6B2). These clones resulted in either no obvious alterations or only very slight perturbations of the normal axon patterns. It was not possible to determine if the *ph* neurons of a small clone actually extended axons in the apparently wild-type axon ladder which developed nearby.

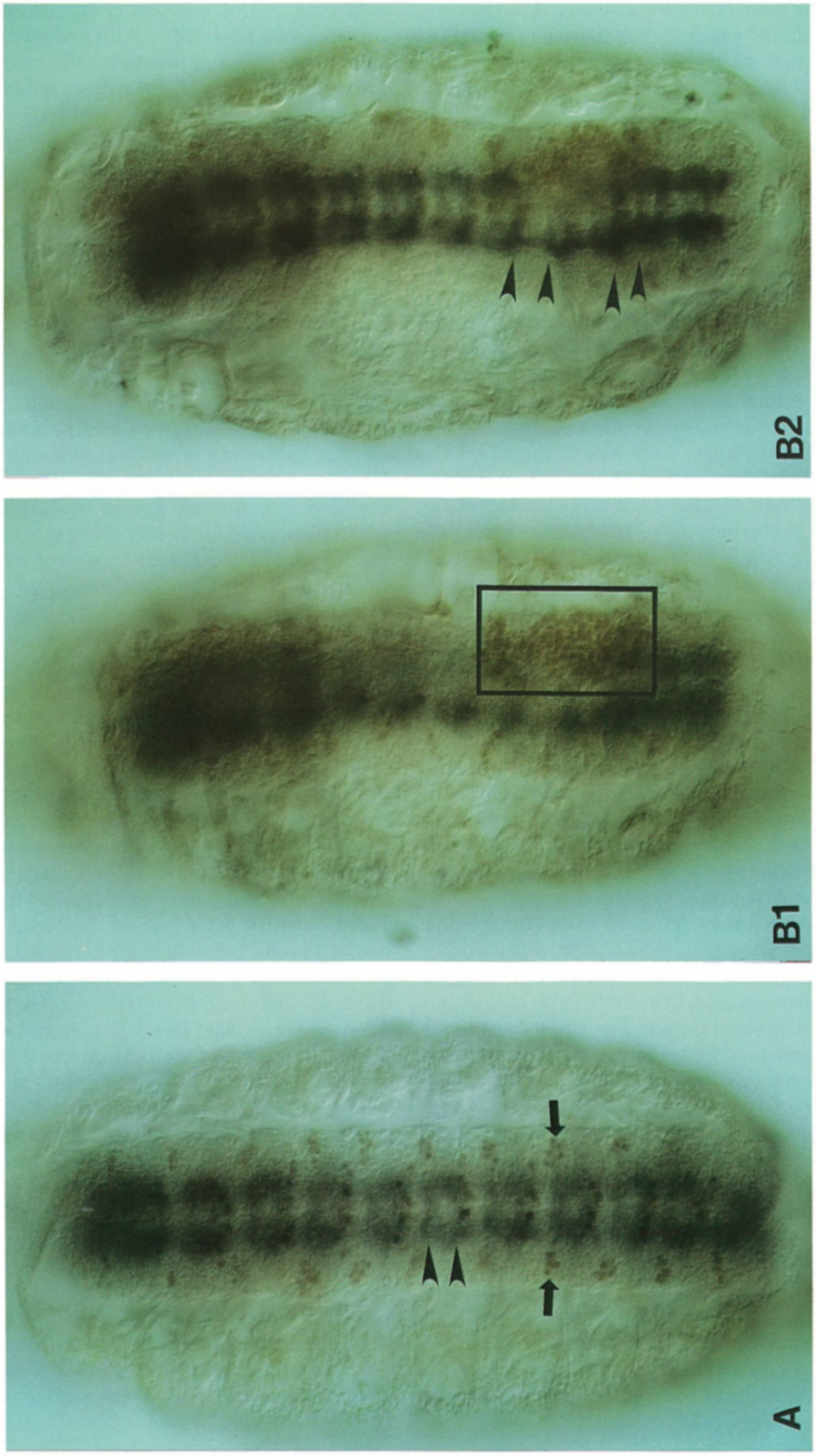
### *Polyhomeotic Neurons Extend Normal Axons in Tissue Culture*

It has previously been proposed (Smouse *et al.*, 1988) that the *ph* axonal phenotype may be due to a drastic loss or change in neuronal cell identity or to the absence of a substrate or growth factor required for normal axonogenesis. Observations have provided indirect evidence that the phenotype is not simply caused by the inability of mutant neurons to extend axons, i.e., because of a cell-autonomous defect in axon structure or elongation. As a direct experimental test of the ability of *ph* neurons to extend axons, 4- to 6-hr-old embryos were manually disrupted and the cells from single embryos were plated on coverslips and cultured *in vitro* for at least 24 hr in media which had been conditioned by wild-type embryonic cells (see Materials and Methods). It has been previously shown that such disrupted and cultured embryonic cells undergo division and differentiation much as they would *in vivo* (Furst and Mahowald, 1985). Since it is not possible to identify *ph* embryos from wild-type at 4–6 hr of development, the cells were fixed after culture and stained with an anti-*Ubx* antibody to distinguish those coverslips on which cells from *ph* embryos had been plated. If 90% of the cells on a given coverslip failed to express *Ubx*, it was assumed that that coverslip bore cells from a *ph* embryo. Under these conditions, no differences in axon length or complexity of axon branching could be seen between the wild-type and *ph* cultures (Figs. 7B and 7D). These results suggest that the inability of *ph* neurons to extend axons *in vivo* is not due to a cell-autonomous defect in the axons themselves, but rather is due to the context in which those axons are extended.

## DISCUSSION

The loss of *ph* gene function during embryogenesis results in a complex phenotype that includes death of the ventral epidermis, abnormal patterns of gene expression, and an abnormal pattern of axonogenesis in the central nervous system (Dura *et al.*, 1987; Smouse *et al.*, 1988; Dura and Ingham, 1988). We have attempted to dissociate these aspects of the *ph* phenotype through the use of clonal analysis and double mutant combinations. It is important to determine whether death of the ventral epidermis is cell autonomous or whether, for instance, it is dependent on the defects associated with the CNS. It is already known that neuroblasts interact with epidermal cell precursors early in development to inhibit them from assuming neuroblast fates (Doe and Goodman, 1985; Campos-Ortega, 1988). In a similar fashion, epidermal cell viability may depend on later interactions between these two tissue types; these interactions may require wild-type *ph* gene expression. We can also ask if the death of these cells is dependent on cell fate or if it depends on their position in the embryo.

Cell death caused by loss of *ph* function is spatially restricted to the epidermal cells from the neurogenic region. We have not yet identified any other cell or tissue type which also undergoes massive death in *ph* embryos. Ventral epidermal cell death is apparently cell autonomous and cell-cycle dependent, since clones induced early in development die, while clones induced two cell cycles prior to their terminal division survive. The most likely interpretation of this result is that there is sufficient perdurance of wild-type *ph* gene function for at least two cell cycles following the induction of homozygous mutant clones. It is still possible, however, that the survival of small *ph* clones is due to local rescue of mutant cells by direct contact with wild-type cells. This type of local nonautonomy has been reported for *Notch* mutations (Technau and Campos-Ortega, 1987). Two lines of evidence suggest that it is perdurance which rescues the mutant cells. First, the induction of *ph* clones during larval development also demonstrates that perdurance of *ph* gene product permits survival of mutant cells for several divisions (Santamaria *et al.*, 1989). Second, cell-cycle dependence of viability is also observed in *ph;stg* double mutant embryos, in which the arrest of cell division caused by loss of *stg* rescues the ventral epidermal cells from death.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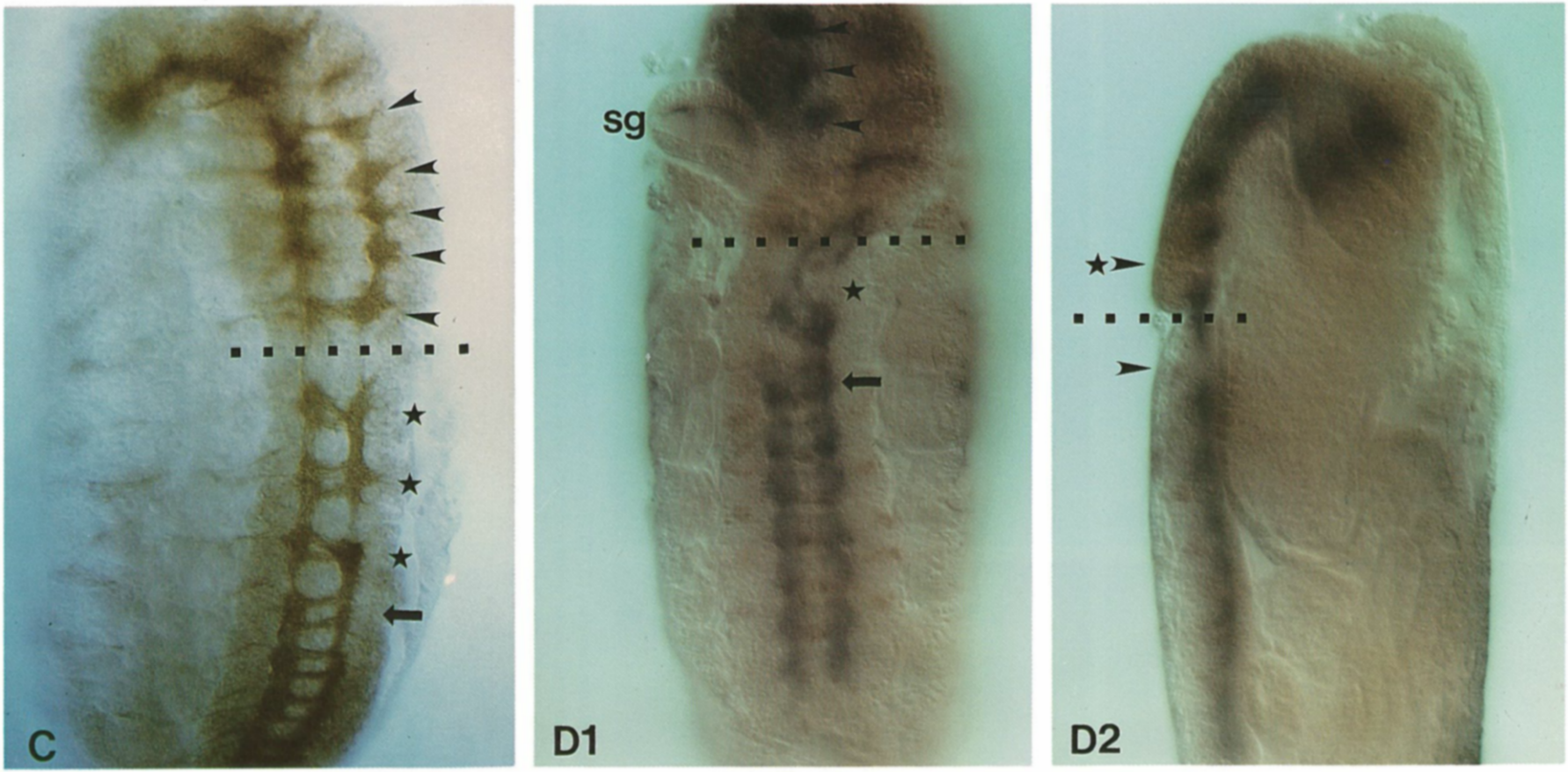


FIG. 6. Mosaic analysis of *polyhomeotic* central nervous system. In all panels, anterior is up. (A) Wild-type embryo stained with anti-*eve* (brown nuclei; indicated by the arrows) and anti-BP102 (blue axons; the arrowheads point to the anterior and posterior commissures of a single segment). (B1, B2) Two focal planes of a mosaic embryo double-labeled with the same antibodies. A small clone of hemizygous, *ph* neurons (box) express *eve* ectopically and are surrounded by wild-type neurons. The more dorsal focal plane in (B2) shows that the global pattern of axon fascicles (arrowheads) is normal, even in the vicinity of the clone. The staining of the longitudinal connective that runs through the clone is obscured by the intense *eve* staining, but the presence of the longitudinal in this region was confirmed under Nomarski optics. (C) A mosaic embryo labeled with anti-HRP and containing a large *ph* clone anterior to the dotted line. The ventral epidermis associated with the clone has died and the axons originating from neuromeres within the clone form "*ph* bundles" (arrowheads). The axon fascicles in presumed wild-type neuromeres immediately posterior to the clone (stars) do not have completely normal morphology, but do not resemble "*ph* bundles." Neuromeres which are three segments posterior to the clone (arrow) have normal axon morphology. (D1, D2) Ventral (D1) and lateral (D2) views of a mosaic embryo double-labeled as in (A). The dotted line marks the posterior boundary of the mutant tissue. The *ph* neurons express *eve* ectopically and form "*ph* bundles" (arrowheads), while the immediately adjacent wild-type neurons (star) express *eve* in a normal pattern, but do not form normal axon fascicles. The normal axon ladder (arrow) appears two segments posterior to the clone. In the lateral view (D2), the death of the ventral epidermis associated with the clone is clearly visible (star), while the adjacent wild-type epidermis (arrowhead) remains viable. The salivary gland (sg) protrudes from the embryo shown in (D1).

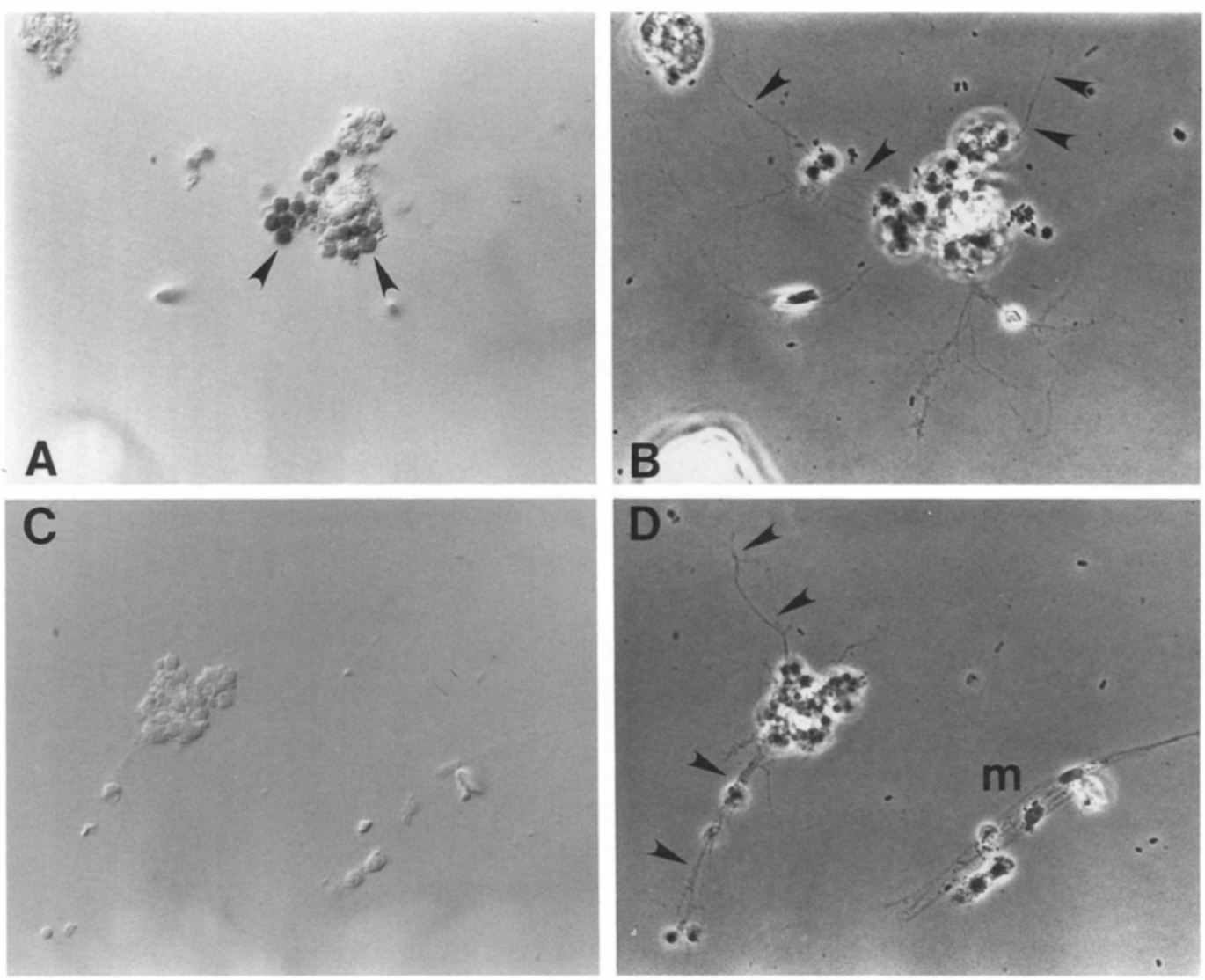


FIG. 7. *In vitro* culture of wild-type and *polyhomeotic* neurons. (A, B) *In vitro* cultured neurons derived from a single wild-type embryo, stained with anti-*Ubx*, and photographed under Nomarski (A) or phase-contrast (B) optics. Note the neuronal nuclei expressing *Ubx* (arrowheads in A) and the axonal projections of these cells (arrowheads in B). (C, D) *In vitro* cultured neurons derived from a single *ph* embryo. There is no *Ubx* expression (C), but the axonal projections (arrowheads) approximate those of wild-type in length and complexity. A differentiated muscle is also visible (m).

Rescue in this case is presumably mediated by maternally supplied wild-type *ph* gene product (Smouse *et al.*, 1988).

Ventral epidermal cell death is also dependent on the dorsal/ventral identities of the cells, since epidermal cells outside the neurogenic region do not die, and since the extent of cell death responds to changes in the dorsal/ventral axis of the embryo. Thus, when the fate of the ventral epidermal cells is changed from ventral to dorsal by mutations at the *dorsal* locus, they are rescued from cell death. When the fates of all epidermal cells are ventralized by gain-of-function mutations at the *Toll* locus, the zone of death is expanded to include most of the epidermis.

Mutations which perturb the dorsal/ventral axis of the embryo act very early in development, prior to sorting out of nervous system and epidermal lineages. Thus, while these mutants indicate that *ph* responds to the determinants of the dorsal/ventral axis, they do not directly address the question of whether the cell death is not in some way related to the concurrent defects in the CNS. However, embryos lacking zygotic *ph* and maternal and zygotic *pcx* gene expression do not exhibit death of the ventral epidermis; there is essentially no ventral epidermis in these embryos since all presumptive epidermal cells of the neurogenic region have assumed the fate of neuroblasts. The progeny of these transformed neuroblasts are viable and form axon

bundles as in embryos mutant only for *ph*. The absence of neuroblast or neuronal cell death in these double mutants further supports the notion that cell death is specific to the ventral epidermal fate, and not "ventral position" specific, since cells whose fate is changed from epidermis to neuroblast survive.

These results support the conclusion that it is the identity of the ventral epidermal cells which leads to their death in the absence of zygotic *ph* expression. This identity is dependent on the determinants of the dorsal/ventral axis and presumably on interactions with neuroblasts. It is not known how the differences in identity between ventral, neurogenic epidermis and lateral/dorsal epidermis are manifested, and how these differences could lead to such different responses to the mutation. It could be, for instance, that there is an intrinsic and nonuniform requirement for *ph* function in epidermal cells, with epidermal cells from the neurogenic region requiring *ph* function in greater amounts or for a longer period of time than all other epidermal cells. Instead, it could be that there is a uniform requirement for *ph* in all epidermis and that there is some secondary aspect of neurogenic ectoderm-derived epidermis which makes it more sensitive to loss of *ph* function. This idea is supported to some extent by the observation that *ph* is required for the survival of all imaginal epidermal cells (Santamaria *et al.*, 1989). Secondary aspects of neurogenic epidermis development could include nonuniform distribution of *ph* gene product, increased numbers of cell divisions, or a combination of cell divisions and segregation of, or interactions with, the neuroblasts that cause a relatively greater depletion of *ph* function.

The loss of *ph* also has severe effects on the development of the embryonic nervous system. Two discrete CNS phenotypes are readily apparent (Smouse *et al.*, 1988): first, there is a general misregulation of expression of developmentally important genes in the CNS. This includes the ectopic, overexpression of one gene, *even-skipped*, and the repression of many other homeotic and segmentation genes. Second, there is a dramatic axonal phenotype characterized by the formation of "*ph* bundles." To better understand the origin of these CNS phenotypes, we have addressed the following questions: Is either CNS phenotype related to epidermal cell death? Are the two CNS phenotypes cell autonomous? Are the changes in gene expression separable from the axon phenotype?

In *stg;ph* double mutants, there is no epidermal cell death, yet the changes in gene expression in the CNS which are appropriate for *ph* clearly occur. The expression of *Ubx* is repressed and the expression of *eve* is derepressed in virtually all neuroblasts. Thus, this aspect of the CNS phenotype is separable and independent of cell death; it is not rescuable by perdurance of maternally supplied *ph* gene product. The embryos doubly mutant for *ph* and *pcx* indicate that epidermal cell death is also not required for the formation of the abnormal axon bundles which are characteristic of *ph*. In these double mutants, the extra neurons send their axons into the "*ph* bundle" appropriate for their hemiganglion. It should also be noted that it is not simply the loss of the ventral epidermis which is responsible for inducing these phenotypes, since embryos mutant only for *pcx* also lack ventral epidermis but do not display the *ph*-specific phenotypes.

These experiments demonstrate the independence of the epidermal phenotype and the CNS phenotypes. Thus, *ph* is required in two lineally related tissues with strikingly different consequences for each tissue. While these experiments rule out cell death as being causal for the CNS phenotypes, they do not, however, address directly whether the CNS phenotypes are cell autonomous, since they may be the result of interactions between neurons or between neurons and some other cell type. It seems likely that the misregulation of gene expression in the CNS is indeed cell autonomous—the clones of mutant neurons observed in the gynandromorphs exhibit the *ph*-specific patterns of *Ubx* and *eve* expression. Thus, the mere presence of these clones argues against long-range cell interactions being involved in establishing patterns of gene expression in the CNS. Furthermore, the repression of *Ubx* expression in isolated *ph* neurons cultured in media conditioned by wild-type embryos argues against a role for secreted, diffusible factors in determining this aspect of the CNS phenotype. There is, however, no independent way of assessing the genotypes of the clones or the surrounding neurons to rule out some sort of local nonautonomy, which might be the result of direct cell-to-cell contacts.

The cell autonomy of the axonal phenotype is not completely resolved. It is known that the complex fasciculation patterns of wild-type neurons depend on equally complex interactions between neurons and glia, extracellular matrix, and other neurons. Whole segments of CNS which are mutant for *ph* exhibit a fully penetrant *ph* axonal phenotype. Furthermore, the adjacent wild-type segments are also affected, showing an axonal phenotype that is disorganized, but clearly distinct from that of *ph*. This suggests that completely separated segments mutant for *ph* behave autonomously with respect to the axonal phenotype and can affect nearby wild-type segments which would normally receive axonal inputs from the neighboring *ph* segments. Small clones of *ph* neurons do not appear completely normal, nor are they fully penetrant for the axonal phenotype. They do not grossly affect the wild-type neurons within the same segment or those of adjacent segments although subtle alterations might not be detectable. We cannot yet determine whether clones

of *ph* neurons extend axons in normal or abnormal patterns when they are surrounded by wild-type neurons or when wild-type axons are within filipodial grasp. However, we can conclude that wild-type neurons do not secrete factors with long-range diffusibility which rescue *ph* neurons, nor do *ph* neurons produce negative factors that inhibit immediately adjacent cells from extending axons in normal pathways. The former result suggests that the cell culture experiments, where *ph* neurons were grown in media conditioned by wild-type cells, are not complicated by the possibility of rescue by secreted products of those wild-type cells. These *in vitro* experiments demonstrate that there is no cell-autonomous defect in the ability of *ph* neurons to extend axons.

These results imply that the axonal phenotype is the result of a defect in the ability of neurons to pioneer and recognize appropriate axonal pathways. It is not clear if this defect is cell autonomous, nonautonomous, or some combination of the two. One reasonable hypothesis to explain the defect proposes that the identities of neurons are perturbed by the drastic changes in homeotic and segmentation gene expression. This "neuronal identity crisis" could cause the abnormal fasciculation of axons from neighboring neurons within the same hemiganglion. If this change or loss of identity were confined to a neuromere or group of neuromeres, as in the case of the gynandromorphs, then the adjacent, wild-type cells, which would normally extend into the mutant segment(s), would instead confront mutant territory. This mutant territory might inhibit pathfinding and fasciculation by the ingrowing axons of wild-type neurons. Similarly, wild-type segments would not receive normal axonal inputs from adjacent mutant neurons, which would tend to cause a disruption of the longitudinal connectives running between segments. In fact, such a disruption of connectives is observed in wild-type neuromeres immediately adjacent to mutant ones. In small clones, which are most likely heterogeneous for mutant and wild-type neurons, there may be sufficient positional cues, either within the clone or within filipodial grasp, to allow the wild-type neurons to traverse the mutant territory in a nearly normal fashion. Thus, the *ph* axonal phenotype may be the cumulative result of autonomous changes in neuronal identity and subsequent changes in the cell interactions that are required for normal axon pathway recognition. The conclusion that changes in neuronal identity can result in changes in axon morphology has also been obtained for neuron-specific phenotypes associated with *even-skipped* mutations (Doe *et al.*, 1988). A critical test of the model proposed for the *ph* phenotype will be to determine if there is indeed a causal relationship between the observed changes in gene expression and the axonal phenotype.

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