Development 121, 3713-3721 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995
DEV7431

Cell fate specification by *even-skipped* expression in the *Drosophila* nervous system is coupled to cell cycle progression

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SUMMARY

The correct specification of defined neurons in the *Drosophila* central nervous system is dependent on *even-skipped*. During CNS development, *even-skipped* expression starts in the ganglion mother cell resulting from the first asymmetric division of neuroblast NB 1-1. This first division of NB 1-1 (and of the other early neuroblasts as well) is temporally controlled by the transcriptional regulation of *string* expression, which we have manipulated experimentally. *even-skipped* expression still occurs if the first neuroblast division is delayed, but not if the division is prohibited. Moreover, *even-skipped* expression is also dependent on progression through S phase which follows immediately after the first division. However, cytokinesis during the first NB division is not required for *even-skipped* expression as revealed by observations in *pebble* mutant embryos. Our results demonstrate therefore that *even-skipped* expression is coupled to cell cycle progression, presumably in order to prevent a premature activation of expression by a positive regulator which is produced already in the neuroblast during G_2 and segregated asymmetrically into the ganglion mother cell during mitosis.

Key words: *even-skipped*, nervous system development, neuroblast, cell cycle, mitosis, S phase, cell specification, *string*, *Drosophila*

INTRODUCTION

The formation of the nervous system in higher eukaryotes requires precise specification of many different cell types at the correct time and position. Extensive analyses in *Drosophila* have provided substantial insight into this complex developmental process (reviewed by Campos-Ortega, 1993; Goodman and Doe, 1993). The development of the central nervous system (CNS) is initiated in a monolayer epithelium (the neurogenic region) which is subdivided into small equivalence groups (the proneural clusters). Individual neuroblasts, the founder cells of the nervous system, are singled out from these proneural clusters in a process involving lateral inhibition whereby neighbouring cells are inhibited from acquiring neural fates. The neuroblasts then delaminate in a stereotyped, segmentally repeated pattern. Each neuroblast is characterized by its specific position and time of delamination (Doe, 1992). By asymmetric cell divisions, the neuroblasts subsequently generate a series of ganglion mother cells (GMC) which divide once more to produce neurons and in some cases glial cells (Udolph et al., 1993; Taghert and Goodman, 1984).

The neuroblast lineages are largely invariant and cell fate specification within the lineages is highly correlated with birth order. Specific neurons are always derived from a defined neuroblast and originate from a GMC generated after a distinct neuroblast division (Taghert and Goodman, 1984; Thomas et al., 1984; Doe, 1992). Experimental analyses in grasshopper and *Drosophila* have indicated that neuroblast-intrinsic mechanisms are responsible for the specification of cell fate according to birth order (Taghert and Goodman, 1984; Taghert et al., 1984; Doe and Goodman, 1985; Huff et al., 1989; Udolph et al., 1993). A particularly well-studied example is the specification of the two pioneering neurons designated aCC and pCC. These neurons originate from the first GMC generated from neuroblast (NB) 1-1 (Thomas et al., 1984; Goodman et al., 1984). Transplantation experiments have clearly shown that the specification of these neurons and other cell types in the lineage of NB 1-1 occurs independent of the environment (Prokop and Technau, 1994; Udolph et al., 1993; Doe and Goodman, 1985). Furthermore, aCC and pCC are missing after ablation of the first GMC and cannot be replaced by the progeny from the second GMC2 demonstrating the absence of regulation within the lineage (Doe and Goodman, 1985; Goodman et al., 1984). The molecular mechanisms of this lineage-intrinsic fate specification are not clear. However, a coupling of cell fate specification and cell cycle progression is an attractive possibility.

The molecular mechanisms co-ordinating cell cycle progression and other developmental processes have been analyzed in considerable detail in *Drosophila*, but not in the neuroblast lineages. The mechanisms controlling cell cycle progression during embryogenesis vary depending on developmental stage and tissue (Orr-Weaver, 1994; Lehner, 1994). The first thirteen, syncytial cycles of alternating S phases and M phases are controlled exclusively by post-transcriptional mechanisms.

Following cellularization, a G_2 phase is added during cell cycle 14 and entry into mitosis 14 is precisely controlled in a defined spatial and temporal pattern by transcriptional control of zygotic *string* (*stg*) expression (Foe, 1989; Edgar and O'Farrell, 1989, 1990). *stg* encodes a cdc25-phosphatase which specifically activates the mitosis promoting cdc2-kinase in complexes with the regulatory cyclins A or B. Subsequent divisions in the epidermis are also spatially and temporally regulated by pulses of *stg* expression. The large regulatory region of the *stg* gene is composed of a number of modular enhancers, which control partial aspects of the complex expression pattern and which appear to be targets of the transcriptional regulators that define positional information and developmental fate in the *Drosophila* embryo (Edgar et al., 1994).

In contrast to the precise *stg*-dependent control of entry into mitosis, entry of epidermal cells into S phase always occurs immediately after each mitosis, without an intervening G_1 phase. After the final mitosis, however, the epidermal cells enter into an extended G_1 phase for the first time. Entry into this postmitotic G_1 phase is dependent on downregulation of cyclin E which associates with the cdc2c kinase and is known to be essential for entry into S phase (Knoblich et al., 1994). The time of cyclin E downregulation and exit from proliferation is dependent on cell fate. Developmental control of the G_1/S transition, therefore, comes into play only at the very end of the epidermal proliferation.

In contrast to the well-studied epidermis where fewer cell types are specified with lower spatial resolution, CNS development might require a more elaborate regulation of cell cycle progression. In fact, additional regulation at the G_1/S boundary has been suggested to occur during the development of the peripheral nervous system (Richardson et al., 1993). However, we have carefully analyzed the cell cycle progression of the early neuroblasts and find the same characteristics as in the dermoblasts. The first neuroblast division is preceded by String accumulation and followed by an immediate entry into S phase consistent with the continuous expression of cyclin E.

The identification of the crucial cell cycle regulators provides the basis for experiments addressing the coupling of cell cycle progression and cell fate specification during neurogenesis. We show here that *even-skipped* (*eve*), which produces a homeodomain-containing transcription factor (Eve) that is known to be required for the correct specification of the aCC neuron (Doe et al., 1988b), is not expressed in the CNS of *string* mutants, where all cells arrest in G_2 phase of cycle 14. Manipulating the timing of *stg* expression indicates that the timing of cell divisions is not critical for *eve* expression. Additional experiments involving inhibition of either S-phase or cytokinesis further demonstrate that *eve*-dependent cell fate specification in the nervous system is clearly coupled to cell cycle progression.

MATERIALS AND METHODS

Fly strains

For initial analysis of Eve expression in *string* mutants, the strong alleles stg^{7B} and stg^{8A} (Jürgens et al., 1984; Edgar and O'Farrell, 1989) were used. As no differences were detected, stg^{7B} was used for all further analyses.

The $P[w^+, Hs\text{-}stg3B]$ insertion (Edgar and O'Farrell, 1990) was recombined with stg^{7B} and balanced over *TM3*, *Sb* $P[w^+, Ubx\text{-}lacZ]$ allowing the identification of homozygous stg^{7B} $P[w^+, Hs\text{-}stg3B]$ progeny due to the lack of *lacZ* expression.

The *pbl* alleles pbl^{7O} and pbl^{11D} have been described previously (Lehner, 1992; Hime and Saint, 1992). For all experiments, both alleles or transheterozygotes were used.

The following alleles of *Pc-G* genes were used in combination with stg^{7B} in our experiments: Pc^{9M21}, Psc^{IIN48}, Pcl^{XM3} and Asx^{II75}, and a chromosome containing both Asx^{XF23} and Pcl^{XM3}.

Bromodeoxyuridine-labeling, immunolabeling and in situ hybridization

Pulse-labeling with 5-bromodeoxyuridine (BrdU) was done either after permeabilization of the vitelline membrane with octane as described previously (Lehner et al., 1991) or by microinjection of BrdU (see below). Immunofluorescent detection of incorporated BrdU was carried out as described previously (Lehner et al., 1991).

Additional immunolabeling experiments were done essentially as described (Lehner et al., 1991; Kania et al., 1990) with affinity-purified rabbit antibodies against *Drosophila* Cyclin B3 (H. Jacobs and C. F. L., unpublished data), against String (kindly provided by B. Edgar, Fred Hutchison Cancer Research Center, Seattle), against Hb (kindly provided by P. MacDonald, Stanford University) and against Eve (kindly provided by M. Frasch, Mount Sinai School of Medicine, New York), and with the mouse monoclonal antibodies 22C10 (kindly provided by W. Chia, Institute of Molecular and Cell Biology, Singapore), against En (kindly provided by J.-P. Vincent, MRC Cambridge) and against β-Galactosidase (Promega). Secondary goat anti-rabbit antibodies were either coupled to alkaline phosphatase (AP) or horse radish peroxidase (PO) and were detected as described previously (Kania et al., 1990).

For double-labeling with anti-Eve antibodies and 22C10 or anti-Eve and anti-En, the primary antibodies were applied simultaneously, followed by washes and an incubation with a mixture of AP-conjugated anti-rabbit and PO-conjugated anti-mouse antibodies. AP and PO colour reactions were carried out successively as described by Kania et al. (1990). Immunocytochemically labelled embryos were mounted (in some cases after prior dissection) in 70% glycerol.

For double-labeling with anti-Eve and anti-BrdU antibodies, anti-Eve-labeling using a fluoresceine-conjugated secondary antibody was carried out before HCl-treatment and anti-BrdU-labeling using a rhodamine-conjugated secondary antibody. Immunofluorescently labelled embryos were mounted in PBS conaining 70% glycerol, 10 mg/ml propylgallate and 0.5 mg/ml phenylenediamine. Immunofluorescent signals obtained in double-labeling experiments were recorded with a cooled CCD camera (Photometrics) and merged using Adobe Photoshop software.

To generate probes for in situ hybridization, we used a 1 kb *Pst*I genomic fragment containing most of the *eve* coding region (Frasch et al., 1987). The cyclin E probe has been described in Knoblich et al. (1994). In situ hybridization was carried as described by Tautz and Pfeifle (1989) and Knoblich et al. (1994).

Injection experiments

Embryos were microinjected with BrdU (10 mg/ml), or aphidicolin (4 mg/ml, diluted from a 20 mg/ml stock in dimethylsulfoxide), or 20% dimethylsulfoxide in control experiments. For accurate staging, we collected embryos at stage 6 according to Campos-Ortega and Hartenstein (1985). These embryos were further aged at 18˚C for defined periods. Embryos aged for 2 hours beyond stage 6 were used for microinjection before or during the S phase which follows after the first division of NB 1-1 in GMC 1-1a (see Results). Embryos aged for 3.5 hours beyond stage 6 were used for microinjection at the end or after this S phase. After injection, embryos were aged for an additional 4-5 hours at 18˚C before fixation and manual devitellinization.

RESULTS

Timing and regulation of the first neuroblast division

To investigate the interdependence of cell cycle progression and cell fate determination, we first analysed the timing and regulation of the first neuroblast divisions. We used antibodies against cyclin B3 to monitor these divisions. Cyclin B3 accumulates during the cell cycle and is rapidly degraded during mitosis like cyclins A and B (Lehner and O'Farrell, 1989, 1990; Whitfield et al., 1990; H. Jacobs and C. F. L, unpublished observation). In contrast to cyclins A and B, cyclin B3 accumulates in the cell nucleus. Anti-cyclin B3 labeling can therefore be assigned more easily to individual cells.

After the last syncytial division (mitosis 13), all nuclei at the egg surface enter immediately into S phase of cycle 14 and become cellularized. Concomitantly also cyclins A, B and B3 accumulate, and the progression through the next division (mitosis 14) is accompanied by the rapid degradation of these cyclins. Cyclin B3 degradation in the neuroblasts delaminating in the first two waves (SI and SII; Doe, 1992) is observed only after their delamination (Fig. 1A,B) indicating that these neuroblasts delaminate in the G_2 phase of cycle 14. The degradation of cyclin B3 in these neuroblasts and hence mitosis 14 occurs in a reproducible pattern. The neuroblasts in the lateral row and NBs 5-2 and 5-3 divide first (Fig. 1B).

Interestingly, the pattern of the neuroblast division is anticipated precisely by the pattern of *stg* expression as described previously in the case of the epidermis (Edgar and O'Farrell, 1989). *stg* mRNA and protein accumulation is first observed in the neuroblasts, which divide first (Fig. 1C, data not shown). *stg* mRNA and protein were found to disappear rapidly from the neuroblasts after the division (data not shown).

Pulse-labelling with BrdU revealed that the neuroblasts enter S phase of cycle 15 immediately after mitosis 14 (Fig. 1D). The pattern of BrdU incorporation corresponds precisely to the pattern of *stg* expression and of cyclin B3 degradation (compare Fig. 1D with 1B,C) indicating that there is at most a very short G_1 phase. Consistent with the immediate entry into S phase, cyclin E mRNA (Fig. 1G) and protein (not shown) was found to be present continuously throughout the early neuroblast cycles. Strong cyclin E expression was observed in all neuroblasts except for MP2 (Fig. 1G, arrows) which is characterized by its unique division pattern (see below).

The pattern of the first division of the SI and SII neuroblasts as revealed by BrdU incorporation is illustrated in Fig. 1D-F. Similar results were recently described in an independent study (Hartenstein et al., 1994). The divisions of the neuroblasts in the lateral row and of NB 5-2 and 5-3 which are observed first (Fig. 1D) are followed by the divisions of NB 7-1 and 2-3 (Fig. 1E). Subsequently, additional neuroblasts of the medial row divide followed by divisions in the intermediate row (Fig. 1F). The last cell to divide is MP2, a midline precursor which, unlike all other neuroblasts, undergoes only one symmetric division after delamination (Doe, 1992; Doe et al., 1988a). BrdU incorporation in MP2 therefore is not observed. With the exception of MP2, the pattern of the first neuroblast division appears to occur in the pattern of the preceding delamination. A minor difference might exist between the pattern of delamination and division in the case of the lateral and medial neuroblast rows. While delamination

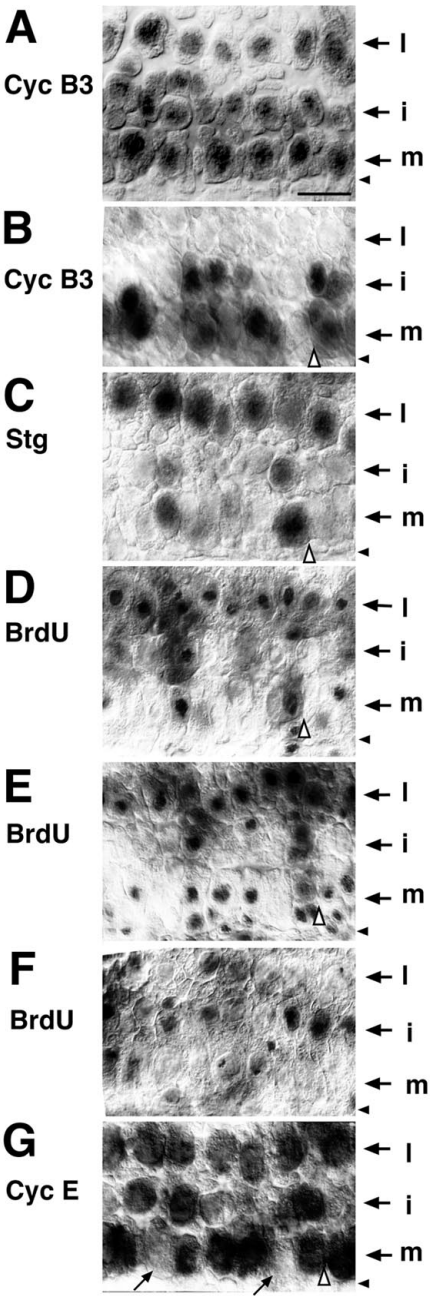


Fig. 1. The timing and regulation of the first neuroblast divisions. Embryos were either first pulse-labelled with BrdU (D-F) or fixed immediately (A-C, G) before immunolabeling with antibodies against cyclin B3 (A,B: Cyc B3), String (C: Stg) and BrdU (D-E: BrdU) or before in situ hybridization with a cyclin E probe (G: Cyc E). High magnification views of the focal plane with the neuroblasts are shown with anterior being oriented to the left and the midline (black arrowheads on the right margin) to the bottom. The lateral (l), intermediate (i) and medial (m) rows of neuroblasts are indicated on the right margin. The location of the parasegmental furrow is indicated by white arrowheads. (A) Cyclin B3 is present in all SI and SII neuroblasts shortly after delamination. (B) At a slightly later stage, cyclin B3 is degraded in the neuroblasts which have already gone through the first division. (C) The neuroblast divisions are anticipated by the pattern of String expression. (D-F) BrdU incorporation starts immediately after the first division reflecting the neuroblast division pattern. (G) Cyclin E RNA is detected in all neuroblasts except for MP2 where only weak signals are present (small arrows). Scale bar in A corresponds to 20 μm.

has been described as being simultaneous in these two rows (Hartenstein and Campos-Ortega, 1984; Doe, 1992), we find that the lateral row divides slightly before the medial row.

The downregulation of cyclin E expression in MP2 after delamination occurs in the G2 phase preceding its final division and is therefore reminiscent of the downregulation that has been observed before the last embryonic cell division in the epidermis (Knoblich et al., 1994). In the epidermis, this downregulation has been shown to be required for the arrest of cell proliferation. Ectopic expression of cyclin E from a heat-inducible Hs-cyclin E transgene immediately after the final division has been shown to result in progression through an additional cell cycle (Knoblich et al., 1994). Although we have not directly tested whether the downregulation of cyclin E is required for the arrest of cell proliferation in the case of MP2, we have observed an increase in the number of *eve*-expressing neurons after Hs-cyclin E expression (data not shown), suggesting that the downregulation of cyclin E is also required to arrest cell proliferation in the neuroblast lineages.

Cell cycle progression is required for *eve* expression in the CNS

To address the role of cell cycle progression for fate specification in the CNS, we analyzed *eve* expression in mutants with an altered cell division programme. In the wild-type CNS, *eve* expression is initiated in two cells per hemisegment at around 5 hours of development (stage 10) (Fig. 2A, see arrows). One of these cells is GMC 1-1a, the GMC generated by the first division of NB 1-1. The other cell is presumably part of the NB 7-1 lineage and not the first GMC (Doe, 1992; T. Bossing and G. Technau, personal communication). GMC 1-1a divides once to give rise to aCC and pCC (Thomas et al., 1984; Goodman et al., 1984). The correct specification of aCC has been shown to be dependent on *eve* expression (Doe et al., 1988b). *eve* expression at later stages is more complex and occurs also in some progeny of NB 4-2 (Fig. 2B,C; Doe, 1992; Doe et al., 1988b).

We analyzed *eve* expression in *stg* mutant embryos. In these embryos, maternal *stg* provisions allow all syncytial divisions. However, zygotic *stg* expression is required for mitosis 14 and, in *stg* mutant embryos, all cells arrest in G_2 of cycle 14 (Edgar and O'Farrell, 1989). Despite this arrest of the mitotic cell cycle

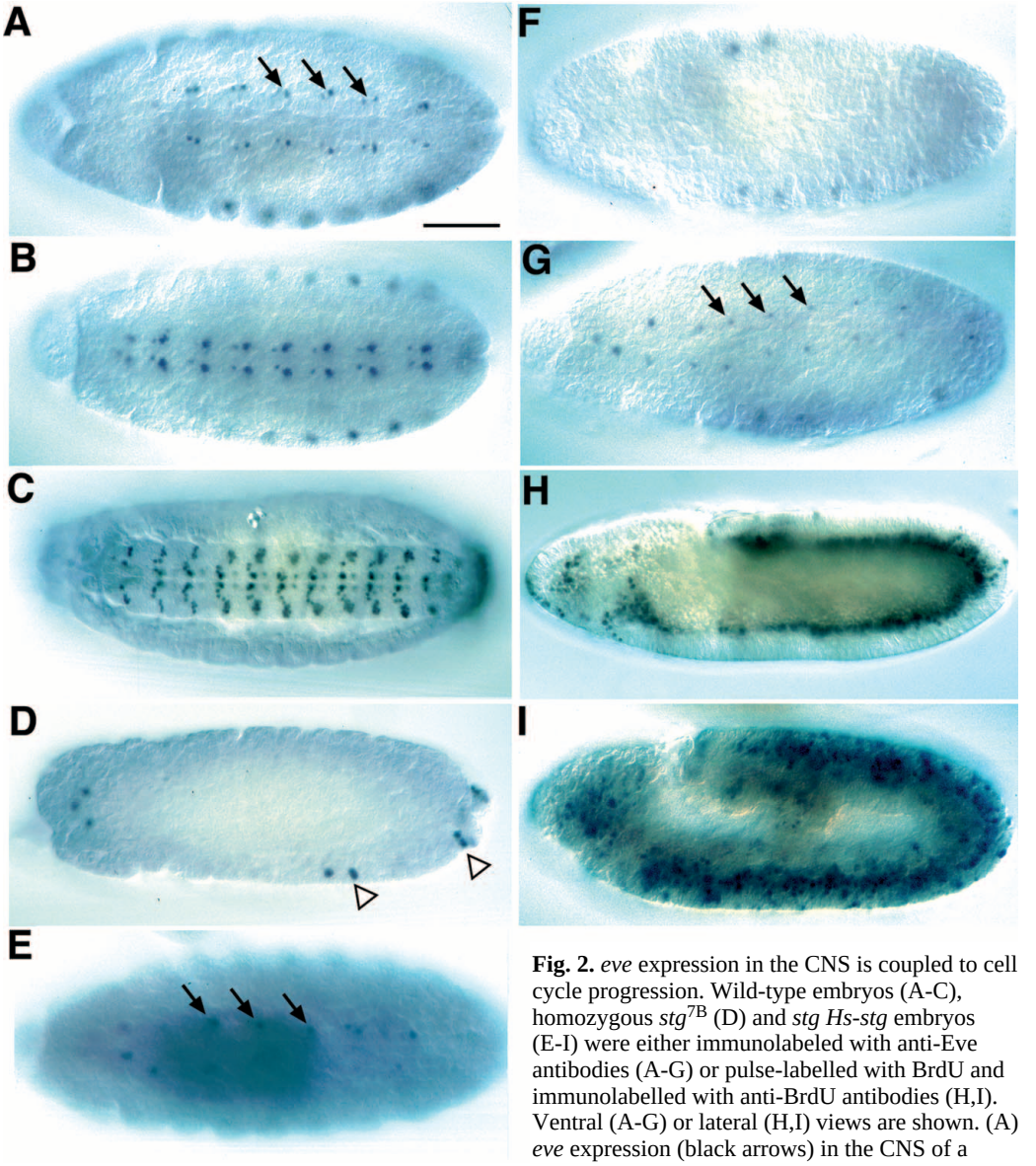


Fig. 2. *eve* expression in the CNS is coupled to cell cycle progression. Wild-type embryos (A-C), homozygous stg^{7B} (D) and *stg Hs-stg* embryos (E-I) were either immunolabeled with anti-Eve antibodies (A-G) or pulse-labelled with BrdU and immunolabelled with anti-BrdU antibodies (H,I). Ventral (A-G) or lateral (H,I) views are shown. (A) *eve* expression (black arrows) in the CNS of a wild-type embryo at stage 10. (B) *eve* expression in the CNS of a wild-type embryo at late stage 11. (C) *eve* expression in the CNS of a wild-type embryo at stage 13. (D) *eve* expression is absent in the CNS of *stg* mutant embryos at stage 13. Eve is only detected in the anal plate and the heart precursors (white arrowheads) and 3 cells located in the anterior. (E) *eve* expression (black arrows) is restored in *stg* mutant embryos at stage 10 after inducing *stg* expression from a *Hs-stg* transgene during a 15 minute incubation at 37˚C followed by 30 minute recovery at 25˚C and fixation. (F) *eve* expression does not occur at stage 10 in *stg Hs-stg* embryos which have not been incubated at 37˚C. (G) A delayed expression of *eve* starting at stage 12 is observed in *stg Hs-stg* embryos which have not been incubated at 37˚C. (H) BrdU incorporation reveals cell cycle progression at stage 9 in the mesoderm of stg Hs-stg embryos which have not been incubated at 37˚C. (I) BrdU incorporation reveals the onset of cell cycle progression at stage 11 in the CNS and the epidermis of *stg Hs-stg* embryos which have not been incubated at 37˚C. Scale bar in A corresponds to 60 μm.

progression, developmental processes continue to a surprising degree in *stg* mutant embryos (Hartenstein and Posakony, 1990). The first two waves of neuroblast delamination that occur before the cell cycle is arrested in *stg* mutant embryos (see above) are not affected. All SI and SII neuroblasts are present in *stg* mutant embryos as revealed by immunolabeling with an antibody against the Hunchback protein (data not shown; see also Hartenstein and Posakony, 1990), a transcription factor expressed early in all neuroblasts (Goodman and Doe, 1993). *eve* expression, however, was never observed in the CNS during the development of *stg* mutant embryos (Fig. 2D) suggesting that neuroblast divisions are required for *eve* expression. In situ hybridization, also failed to reveal *eve* expression in the CNS of *stg* mutants (not shown). In contrast, *eve* expression in the heart precursors and in the anal plate still occurred in *stg* mutant embryos (Fig. 2D, arrowheads).

With a transgene allowing *stg* expression under the control of a heat shock promoter (*Hs-stg*; Edgar and O'Farrell, 1990), we were able to manipulate the timing of *stg* expression and hence the timing of cell divisions in a *stg* mutant background. A heat-induced division at the stage where the SI and SII neuroblasts normally divide for the first time during wild-type development resulted in *eve* expression starting at the normal stage (Fig. 2E). Without the heat treatment, *eve* expression was not observed in the *stg Hs-stg* embryos at this early stage (Fig. 2F).

At later stages, *eve* expression started without prior heat treatment in the *stg Hs-stg* embryos (Fig. 2G). Expression was observed first in a single cell per hemisegment, most likely in GMC 1-1a. The late onset of *eve* expression was preceded by cell divisions that result from basal, heat shock-independent expression from the *Hs-stg* transgene. BrdU pulse labeling revealed that these heat-independent cell divisions occurred in a reproducible pattern. The first divisions were observed in the mesoderm at stage 8/9 (Fig. 2H). At stage 11, divisions started in the CNS and occasional divisions were also seen in the epidermis (Fig. 2I). At this stage, most SI and SII neuroblasts (including NB 1-1) have already progressed through two to three divisions in wild-type embryos. In *stg Hs-stg* embryos, therefore, GMC 1-1a is born at a time where GMC 1-1b or GMC 1-1c are born in wild-type embryos. Despite this delayed birth, GMC 1-1a expressed *eve* and the progeny (also resulting from basal *Hs-stg* expression) differentiated like GMC 1-1a progeny in wild type. Neurons with the characteristic features of aCC were clearly present in *stg Hs-stg* embryos (Fig. 3B, arrowhead). As in wild-type (Fig. 3A; Goodman et al., 1984; Thomas et al., 1984), these neurons were at the characteristic positions next to the posterior commissure, expressed the 22C10 epitope and attempted to project their axons laterally into the intersegmental nerve (Fig. 3B).

Cytokinesis is not required for *eve* expression

The *pebble* (*pbl*) gene is required specifically for cytokinesis (Hime and Saint, 1992; Lehner, 1992). As in *stg* mutants, the onset of phenotypic defects in *pbl* mutants is observed only after cellularization at the stage of mitosis 14, the first real cell division of embryogenesis. In contrast to *stg* mutants, however, in which cell cycle progression is completely blocked before entry into mitosis 14, only cytokinesis is defective in *pbl* mutants. Nuclear divisions occur normally in *pbl* mutants and also further cell cycle progression continues in the absence of cytokinesis.

We labelled *pbl* mutants with anti-Eve antibodies to test whether cell cycle progression in the absence of cytokinesis is sufficient for *eve* expression in the CNS. *eve* expression was clearly observed in the CNS of *pbl* mutants (Fig. 4A). *eve* expression started during stage 10 in cells with two nuclei

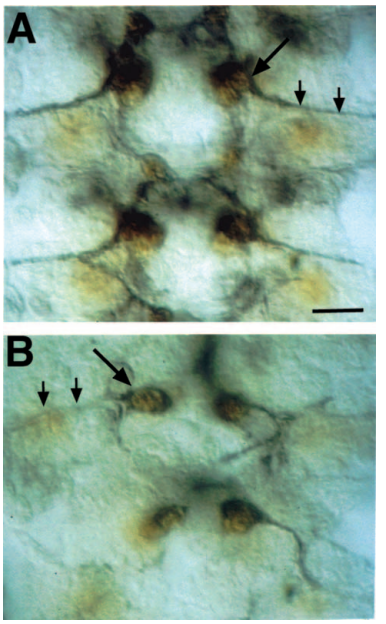


Fig. 3. A delayed division of neuroblast 1-1 does not interfere with the specification of its GMC1 progeny. Wild-type embryos (A) and *stg Hs-stg* embryos (B) at stage 13 were fixed and double-labeled with 22C10 (black) and anti-Eve (brown) antibodies. The cell body of the aCC neuron is indicated by the large arrows and its axon projection by small arrows. The aCC neuron is differentiating in *stg Hs-stg* embryos despite the delayed division of NB 1-1. Bar in A corresponds to 20 μm.

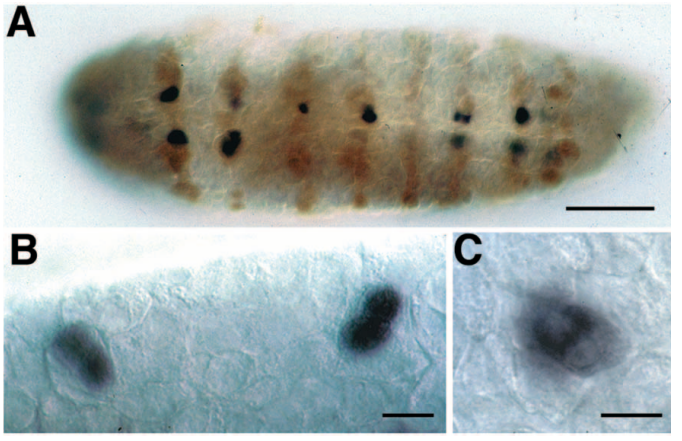


Fig. 4. *eve* expression in the CNS does not require cytokinesis. (A) *pebble* mutant embryos were fixed at stage 10 and double-labeled with anti-Eve (black) and anti-En (brown) antibodies. The ventral view reveals *eve*-expressing cells within the stripe of *en*-expressing cells. High magnification views after anti-Eve immunolabeling (B) or after in situ hybridization (C) reveal that *eve* is expressed in binucleate cells in *pebble* mutant embryos at stage 10. Scale bars in A, B and C correspond to 60, 10 and 10 μm, respectively.

resulting from the failure of cytokinesis during mitosis 14 (Fig. 4B,C).

In most hemisegments, *eve* expression started in a cell corresponding to the NB 1-1A/GMC 1-1a hybrid according to its position (T. Bossing and G. Technau, personal communication) and double-labeling with anti-Engrailed antibodies (Fig. 4A). At locations corresponding to other lineages that express *eve* during wild-type development, expression was not consistently observed in *pbl* mutants. In the lineages where *eve* expression does not start already in the first GMC, the complex multinucleate hybrid cells resulting from progression through repeated cell cycles in the absence of cytokinesis are presumably too abnormal to execute the normal program of *eve* expression. In general, *pbl* mutant embryos become more and more irregular with increasing age in parallel with the cell cycle progression without cytokinesis and many of the cells die at late stages.

Progression through S phase is required for *eve* expression

While *eve* expression in the NB 1-1 lineage is not dependent on cytokinesis, it appears strictly dependent on cell cycle progression according to our analyses in *stg* and *pbl* mutants. Moreover, according to our analysis of the neuroblast division program, it appeared that the expression of *eve* in the first GMC of NB 1-1 started only after it had progressed through S phase. Pulse-labeling with BrdU and double-labeling with anti-Eve antibodies confirmed this notion. We never observed BrdU incorporation in *eve*-expressing cells after labeling during 10 minute pulses (data not shown). By injection of aphidicolin, an inhibitor of DNA replication, we tested whether progression through the S phase that precedes *eve* expression in GMC 1-1a is required for this expression.

Initial experiments indicated that the block of DNA replication resulting from injection of aphidicolin into embryos was not permanent. Therefore, we staged embryos carefully and injected aphidicolin immediately before S phase started in the first GMCs (see Materials and Methods). Subsequently, the embryos were aged to stage 11 when *eve* expression is clearly detectable in wild-type embryos. Control injections without aphidicolin did not inhibit *eve* expression (Fig. 5A). However, injection of aphidicolin resulted in an essentially complete inhibition of *eve* expression (Fig. 5B). When BrdU instead of aphidicolin was injected at the same stage followed by ageing to stage 11, we found that 80% of the *eve*-expressing cells in the CNS had also incorporated BrdU (Fig. 5D). This result clearly demonstrated that the injections were done at a stage before or during S phase occurred in GMC 1-1a. Aphidicolin injections at a slightly later stage (see Material and Methods) did not inhibit *eve* expression (Fig. 5C). BrdU injections indicated that the GMCs had already essentially completed S phase at this later stage, since in 60% of the *eve*-expressing cells BrdU incorporation was not detectable and in 30% of the *eve*-expressing cells BrdU incorporation was only observed in the late replicating heterochromatin (Fig. 5E).

Surprisingly, we found that aphidicolin injections inhibited *eve* expression in the heart precursors, whereas control injections did not. The heart precursors express *eve* in *stg* mutants. Therefore, neither mitosis 14 nor S phase 15 is required for this expression and consequently no inhibition is to be expected from aphidicolin injection. However, control injections of buffer into *stg* and *pbl* mutants also abolished *eve* expression in the heart precursors, but had no effect on *eve* expression in the CNS of *pbl* mutant embryos (not shown). It appears therefore that a cell proliferation arrest (as observed in *stg*, *pbl* or aphidicolin-injected embryos) in combination with injection of DMSO-containing buffer is sufficient for inhibition of *eve* expression in the heart precursor cells.

We emphasize that the inhibition of *eve* expression in the CNS resulting from aphidicolin injections was strictly correlated with the time of S phase in the first GMCs. Inhibition was only observed when aphidicolin was injected before S phase. Moreover, these early injections, which inhibited *eve* expression, did not interfere with *hb* expression in the CNS (not shown). Our aphidicolin injection experiments, therefore, strongly suggest that *eve* expression in the CNS is dependent on progression through S phase.

Polycomb group genes are required for the repression of *eve* expression in the CNS

Pc-G genes are thought to be responsible for a local compaction of chromatin at specific sites (PRE elements; for a review see Pirrotta and Rastelli, 1994; Orlando and Paro, 1995). Binding of Pc-G proteins has been observed in the *eve* region (DeCamillis et al., 1992) and mutations in *Asx*, one of the *Pc-G* genes, were found to result in a mild derepression of expression from an *eve-lacZ* construct (Sinclair et al., 1992), suggesting that the *Pc-G* genes might act as repressors for *eve* expression by local chromatin compaction. S phase might therefore be required for *eve* expression because a remodelling of this repressed chromatin structure might only be possible during the assembly of new chromatin following DNA replication. Therefore, we determined whether mutations in the *Pc-G* genes would allow *eve* expression in the absence of cell cycle progression.

Mutations in various *Pc-G* genes (*Pc*, *Pcl*, *Asx*, *Psc*) were crossed into a *stg* mutant background. While *eve* expression never occurred in the CNS of *stg* mutants, we observed occasional cells expressing *eve* in the CNS of *Asx*; *stg* mutant embryos. In *Asx Pcl*; *stg* mutant embryos more *eve*-expressing cells were observed in the CNS (data not shown). In *Pc stg* mutants, *eve* expression was also observed in the CNS (Fig. 6A). *eve* expression was observed in every *Pc stg* mutant embryo, but not in every segment. The number and position of segments with *eve* expression in the CNS was variable. Moreover, *eve* expression was transient during stage 11 in most of the *Pc stg* embryos. Double-labeling with anti-Hb revealed *hb* expression in the *eve*-expressing cells confirming their neuroblast identity (data not shown). Double-labeling with anti-Eve and anti-En (Fig. 6B and C) indicated that the *eve* expression in the CNS of *Pc stg* mutants most likely corresponds to the expression in the NB 4-2 lineage and clearly not in the NB 1-1 lineage. *Pc* mutations alone had no obvious effect on *eve* expression in the CNS and clearly did not result in ectopic *eve* expression (data not shown). These observations are consistent with the idea that DNA replication is required for *eve* expression to overcome a local chromatin compaction caused by *Pc-G* gene products at least in some of the NB lineages.

DISCUSSION

The results described here demonstrate that the expression of

eve in the CNS, where it is known to be required for the correct specification of neural fates, is strictly coupled to cell cycle progression.

In *stg* mutants, in which the NBs delaminate but never divide to form GMCs, *eve* is not expressed in the CNS. Considering that *eve* is normally expressed only in the GMCs but not in the NBs, this result does not appear to be surprising. However, a previous analysis of *stg* mutant embryos has clearly shown that neuronal differentiation is not blocked despite the cell cycle arrest (Hartenstein and Posakony, 1990). The arrested neuroblasts form axons and express certain neuronal markers (like 22C10) at the correct developmental stage. In addition, differentiation in other tissues also occurs to a surprising extent. The arrest of cell proliferation in *stg* mutants, therefore, is not accompanied by a complete block of later development and the absence of expression (as in the case of *eve*) does not conform to a general rule for late gene expression in *stg* mutants but is rather a rare exception. We suspect, however, that more exceptions will be found in the case of genes specifying cell fates during the progression through the asymmetric divisions characteristic of the neuroblast lineages.

While the induction of a division in *stg* mutants is sufficient to restore *eve* expression in the CNS, we found that this expression is not dependent on the exact timing of the division. Moreover, when the first NB 1-1 division was delayed to the stage at which already the second or third GMC has been generated in wild-type development, the neuron progeny differentiated the characteristics of the first GMC progeny. These observations extend results obtained with cell ablation in grasshopper embryos (Doe and Goodman, 1985). When NB 1-1 was ablated in these experiments, it was replaced by an adjacent cell. The complete NB 1-1 lineage was reinitiated by the replacing cell even when the original NB 1-1 had been ablated only after having already generated the first GMC. Moreover, in additional experiments, when the first GMC was ablated, the corresponding progeny was found to be missing and was not replaced by progeny from the second GMC. While these results strongly suggest that the sequential specification of different cell fates is coupled to progression through the NB 1-1 lineage, they are also consistent with the idea that the sequence of cell fates is specified by a developmental clock which is started at the time of neuroblast formation and which runs independently of cell cycle progression. Our results rule out this interpretation, since NB 1-1 is formed at the normal time in our experiments and only the timing of the divisions is altered.

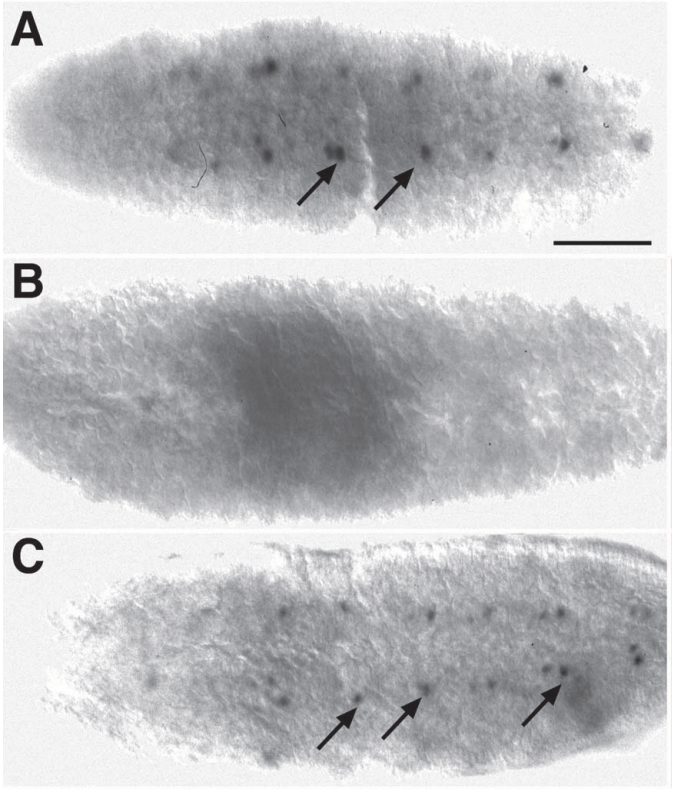
S phase plays a crucial role in the coupling of *eve* expression to cell cycle progression. *eve* expression starts after the GMCs have progressed through S phase and aphidicolin injections before but not after this S phase prevent the expression. Experiments with aphidicolin in *Caenorhabditis elegans* embryos have revealed a similar requirement for S phase in the case of gut lineage marker expression (Edgar and McGhee, 1988). Moreover, cell fate determination in the case of neocortical neurons in mammals has been shown to be correlated with progression through S-phase (McConnell and Kaznowski, 1991). The specific layer to which neurones will migrate is already determined in the neuronal progenitor cells in the ventricular zone but only after progression through S phase.

The observation that *eve* is expressed in the CNS of *stg* mutants if these also carry mutations in *Pc-G* genes, which are crucial for the maintenance of inactive chromatin domains (Pirrotta and Rastelli, 1994, Orlando and Paro, 1995), is consistent with previous experiments which indicate that the *Pc-G* genes are required to maintain repression of homeotic genes even in the absence of DNA replication (Gould et al., 1990). In addition, our results suggest an explanation why progression through S phase is required for the activation of *eve* expression in the CNS. In the course of the chromatin assembly following DNA replication, an inactive *eve* regulatory domain might be remodelled allowing access of transcriptional activators. A role for DNA replication in remodelling chromatin structure has been documented both in vitro and in vivo (Lewin, 1994). A well characterized example has been described recently in yeast where DNA replication was shown to facilitate the reversal of telomeric silencing (Aparicio and Gottschling, 1994). While our observations are consistent with a role for DNA replication in remodelling a regulatory *eve* domain that has been inactivated by *Pc-G* products, a number of different interpretations are not excluded. In particular, we emphasize that de-repression of *eve* in *stg Pc-G* gene double mutants was not observed in all NBs that give rise to *eve*-expressing progeny. Whereas this failure of complete de-repression might be explained by the perdurance of maternally provided *Pc-G* gene products, it is also possible that the *eve* repression in some NBs does not involve the *Pc-G* genes analyzed and DNA replication might be required to overcome the action of different repressors.

While S phase is clearly important for *eve* expression, we do not know whether M phase is also required. At present, it is impossible to suppress the first M phase in NBs experimentally without inhibiting also the subsequent S phase that precedes *eve* expression. However, with the help of *pbl* mutants, we were able to demonstrate that cytokinesis is not essential for *eve* expression. Cell proliferation in *stg* and *pbl* mutants is blocked at the same stage. However, while the cell cycle stops in the G_2 phase in *stg* mutants, it continues in *pbl* mutants in which the cell proliferation arrest results from a failure of cytokinesis (Hime and Saint, 1992; Lehner, 1992). The fact that *eve* is expressed in the CNS of *pbl* mutants therefore further indicates that a cell-intrinsic process dependent on cell cycle progression is involved in the activation of *eve* expression. Similarly, cell cycle progression but not cytokinesis was also found to be required for the differentiation of serotonin-expressing neurons from NBs cultured in vitro (Huff et al., 1989).

Our analyses have important implications for the regulatory mechanisms that confine *eve* expression to GMC 1-1a and prevent expression in the sibling NB 1-1A. The daughter cells, NB 1-1A and GMC 1-1a, both progress through S phase after the first NB division; however, whereas progression through S phase results in *eve* expression in the GMC, it does not in the NB. The observation that *eve* is expressed in the NB 1-1A/GMC 1-1a hybrid cell resulting in the *pbl* mutants strongly argues against the idea that a negative regulator of *eve* expression is segregated exclusively to the NB 1-1A cell during the first asymmetric division in wild-type development. However, all our results are fully consistent with the idea that an activator of *eve* expression is segregated asymmetrically into GMC 1-1a where it is able to access the *eve* regulatory region only after DNA replication (Fig. 7). In this model, the dependence of access on progression through S-phase allows that the activator can be expressed in the NB 1-1 during the G_2 phase without premature activation of *eve* expression before the asymmetric segregation. Genetic analyses have shown that the *prospero* gene product is

an essential activator of the *eve* expression in a number of NB lineages including the NB 1-1 lineage (Doe et al., 1991) and, interestingly, recent observations suggest that Prospero already accumulates in the neuroblasts but does enter the nucleus only after having been segregated asymmetrically into the GMC during the neuroblast division (J. A. Knoblich and Y.-N. Jan, personal communication). Although the functional significance of the asymmetric segregation of Prospero remains to be demonstrated, we emphasize that mechanisms must exist to prevent a premature activation of downstream events whenever cell fate differences are established by the asymmetric segregation of a positive regulator. A mechanism involving a coupling of activation with cell cycle progression as suggested by our analysis in the case of *eve* expression could also operate in other cases.



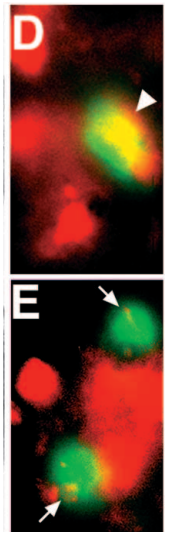


Fig. 5. *eve* expression in the CNS is dependent on progression through S phase. (A-C) Wild-type embryos were injected with buffer (A) or aphidicolin (B,C) and incubated for 4-5 hours at 18˚C before fixation and immunolabeling with anti-Eve. Microinjection was done after ageing beyond stage 6 at 18˚C for 2 hours (A,B) or 3. 5 hours (C). (D,E) BrdU injection at these stages followed by double-labeling with anti-BrdU (red) and anti-Eve (green) indicates that the *eve*-expressing cells in the CNS incorporate BrdU after injection at the earlier stage (D, white arrowhead) but no longer or only in the late replicating heterochromatin (white arrows) after injection at the later stage (E). Ventral views are shown in A-C and high magnification views of CNS regions in D,E. Scale bars in A and D correspond to 60 and 10 μm, respectively.

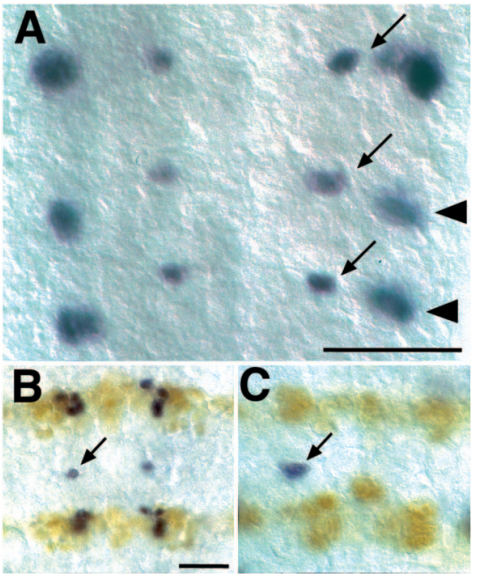


Fig. 6. *eve* expression in the CNS of *Pc-G* mutant embryos occurs independent of cell cycle progression. (A) *Pc stg* double mutant embryos were immunolabeled with anti-Eve at stage 11. The ventral view reveals *eve* expression in the CNS (arrows) and in the heart precursors (arrowheads). (B,C) Double-labeling with anti-Eve (blue) and anti-En (brown) in wild-type (B) and *Pc stg* mutant (C) embryos at stage 11 reveals that *eve* expression in *Pc stg* mutant embryos presumably corresponds to the NB 4-2 and not to the NB 1-1 lineage (arrows). Scale bars in A and B correspond to 60 and 20 μm, respectively.

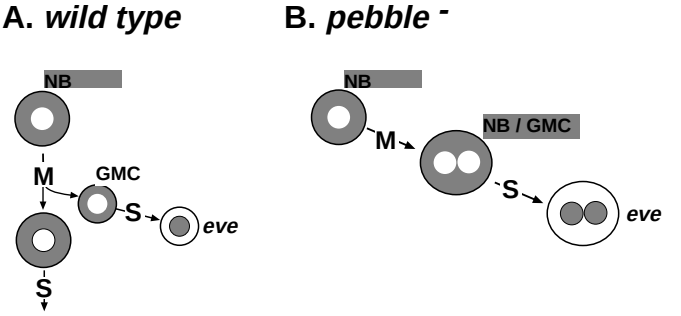


Fig. 7. The coupling of *eve* expression and cell cycle progression. A model for the regulation of *eve* expression in the lineage of NB 1-1 as suggested by our analysis in *stg*, *pbl* and aphidicolin-injected embryos. (A) Wild type: *eve* expression in the first GMC is proposed to be activated by a positive regulator (dark shading) which is already produced in the neuroblast during G_2 before the first asymmetric division. Premature activation of *eve* expression already in the neuroblast is prevented because access to the *eve* regulatory region is dependent on progression through S phase. The lack of access to *eve* regulatory region is symbolized by shading in the cytoplasm, access to the *eve* regulatory region by shading in the nucleus. The positive regulator is segregated asymmetrically to the GMC during mitosis and activates *eve* expression after S phase. (B) *pebble* mutant embryos: the positive regulator gets access to the regulatory region after the NB 1-1A/GMC 1-1a hybrid cell has progressed through S phase. See text for further discussion.

We thank M. Frasch, P. MacDonald, J.-P. Vincent, W. Chia for providing antibodies, T. Bossing, G. Technau and J. Knoblich for communicating results prior to publication, E.-M. Illgen for technical help and the members of our laboratory, in particular M. E. Lane, for comments on the manuscript. In addition, K. W. is indebted to W. Chia for hospitality and support during her stay in his laboratory where the initial observations were made.

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(Accepted 28 July 1995)