J. Genet. Vol. 70, No. 3, December 1991, pp. 161–168. © Printed in India.

In situ patterns of nuclear replication in brain ganglia of *l(2) gl*[4] mutant larvae of *Drosophila melanogaster*

SUJATA ROY and S. C. LAKHOTIA*

Cytogenetics Laboratory, Centre for Advanced Studies in Zoology, Banaras Hindu University, Varanasi 221 005, India

MS received 26 August 1991

Abstract. The *l(2)gl*[4] mutation (a deletion mutation of a recessive oncogene) of *Drosophila melanogaster* causes neuroblastoma in the optic centres of brain of late third instar larvae. We have studied the *in situ* patterns of DNA synthesis in these brains by immunocytochemical detection of cells incorporating 5-bromodeoxyuridine. It was seen that *l(2) gl*[4] brains from younger 3rd instar larvae had fewer replicating cells than in wild type larvae of comparable age but in brain ganglia of older *l(2) gl*[4] larvae the number of replicating cells was much higher. The spatial distribution of replicating cells in optic lobes of brain ganglia of *l(2) gl*[4] larvae was disturbed from early 3rd instar stage, much before the tumourous growth was morphologically detectable. The stereotyped pattern of asymmetrical cell divisions of the neuroblasts and their progeny cells was also not seen in *l(2) gl*[4] brain ganglia. Therefore, it appears that the *l(2) gl*[4] product has an important role early in development to determine the temporal and spatial patterns of neuroblast cell division in developing brains.

Keywords. Antioncogene; BrdU-antibody; neuroblasts; brain tumours.

1. Introduction

The replication programmes of different cell types of *Drosophila* are regulated both temporally and spatially. Accordingly, a given cell enters a mitotic or endoreplication cycle at defined stage of development (see Raman and Lakhotia 1990, for a recent review). DNA synthesis and cell division patterns in larval brain also occur in a programmed manner (Poulson 1950; White and Kankel 1978; Lakhotia and Kumar 1980; Truman and Bate 1988). Most of the neurons of adult brain are formed and differentiated during late larval and early pupal stages (Truman and Bate 1988). The neurons are formed by a stereotyped pattern of cell divisions in which the precursor neuroblasts divide symmetrically to form more neuroblasts which finally divide asymmetrically to give rise to a neuroblast and a ganglion mother cell (GMC). GMCs divide symmetrically to produce pre-ganglion and ganglion cells which differentiate into neurons. The spatial distribution of dividing neuroblasts and GMCs follows a specific pattern during larval period (Truman and Bate 1988).

Immunochemical detection of newly synthesized 5-bromodeoxyuridine (BrdU) substituted DNA in intact tissues provides a convenient approach to examine *in situ* the temporal and spatial patterns of cell divisions (Schubiger and Palka 1987; Truman and Bate 1988). This approach has provided useful information about the spatial locations of neuroblasts and GMCs and their time course of divisions during development of brain in *Drosophila* larvae (Truman and Bate 1988). The reproducibility of the patterns of locations of replicating nuclei in developing brain

*For correspondence

permits detection of any abnormality. In this study, we have compared the *in situ* pattern of location of replicating nuclei in brain ganglia of wild type 3rd instar larvae of *D. melanogaster* with the pattern seen in ganglia of mutant larvae that develop malignant tumours of brain during late 3rd instar stage. Among the several mutant genes that lead to tumourous growth of brain (Gateff and Mechler 1989), one of the better known is the *l(2)gl⁴* and its other mutant alleles. Larvae homozygous for *l(2)gl⁴* recessive mutation appear to grow normally till day 5 (mid or early late 3rd instar stage) after which their brain ganglia begin to show tumourous growth; these larvae fail to pupate but continue as larvae for another 6–7 days during which their body becomes bloated and rather transparent. By this time their brain ganglia also assume irregular morphology. However, up to day 5 or so of larval life, the brain ganglia of these mutant larvae do not display any apparently atypical growth at morphological level. Using the technique of BrdU-labelling and *in situ* detection of nuclei that have incorporated BrdU, we have examined if the regulated pattern of cell divisions is disrupted during or prior to onset of tumourous growth in these mutant larvae.

2. Materials and methods

Larvae of a wild type (Oregon R^+) and the mutant *l(2)gl⁴ or/SM5* stocks of *D. melanogaster* were reared on standard agar–cornmeal–sugar–yeast food at 20°C ± 1°C. The mutant stock generates two types of larvae (for details of the mutants, see Lindsley and Grell 1968), those that are homozygous for *l(2)gl⁴ or* and those which are heterozygous for *l(2)gl⁴ or* mutant alleles (the *SM5* homozygotes do not survive). After the late 3rd instar stage, *l(2)gl⁴* homozygous larvae can be distinguished from heterozygous sibs due to their transparent and bloated body and prolonged larval life. However, at earlier stages they are not easily distinguished. The presence of *or* (*orange*) marker helps in identifying younger *l(2)gl⁴* homozygotes since *or* homozygotes have colourless Malpighian tubules.

Brain ganglia from 4- to 7-day old wild type and *l(2)gl⁴/SM5* and from 4- to 11-day old *l(2)gl⁴* homozygous larvae were dissected in modified Poels' medium (Sinha and Lakhotia 1980) and labelled in dark with 20 mM BrdU (Sigma) at 24°C for 60 min. The labelled ganglia were fixed in 90% ethanol for 30 min, hydrated through descending grades of ethanol and hydrolysed in 2N HCl for 15 min at 24°C. Following hydrolysis, the ganglia were washed several times in PBS (125 mM NaCl, 16·5 mM Na_2HPO_4, 8·5 mM $NaH_2PO_4 . 2H_2O$, pH 7·2) and incubated with 10% goat serum for 1 h at 4°C. After removal of excess of goat serum and washing in PBS, the ganglia were incubated overnight with 1:100 dilution of anti-BrdU antibody (Biocell Consulting, Switzerland) at 4°C in dark. Excess antibody was removed by repeated washings in PBS. Subsequently, the brain ganglia were incubated overnight at 4°C with 1:64 dilution of anti-mouse-IgG-FITC conjugate (Sigma). After the secondary antibody binding, the ganglia were washed several times in PBS and mounted on slides with a solution containing 1:4 PBS:glycerol and 4% *n*-propyl-gallate (Sigma). The whole mount preparations were observed under a fluorescence microscope (Leitz Orthoplan) using 10× oil and 50× oil objective and appropriate fluorescence filter block. At least 7 ganglia of each genotype for each day of development (4 to 7 days for wild type and 4 to 11 days for *l(2)gl⁴* larvae) were examined for the spatial patterns of distribution of BrdU-labelled and fluorescing nuclei.

3. Results

The brain ganglia of wild type 3rd instar larvae have a characteristic shape with two large dorsal cerebral hemispheres and a median ventral ganglion (figure 1). The dorso-lateral sides of cerebral ganglia represent the prominent optic lobes. The size of cerebral ganglia increases to some extent in wild type larvae between day 5 and day 8, when they pupate. The development of brain ganglia of $l(2)gl^4/SM5$ larvae closely followed the patterns in wild type larvae. On the other hand, brain ganglia of 4- and 5-day old $l(2)gl^4$ larvae appeared smaller than those of same age wild type and $l(2)gl^4/SM5$ larvae but showed similar shape (see figure 1a–d). Size of brain in $l(2)gl^4$ larvae increased rapidly from day 6 onwards but the characteristic shape continued till about day 8, after which the brain tissue was seen to be disorganized and to form large irregular mass (figure 1f–g).

Comparison of the overall number of replicating nuclei (which incorporated BrdU and therefore showed immuno-fluorescence) between brain ganglia of wild type and $l(2)gl^4$ larvae, revealed that up to day 5, there were relatively fewer replicating nuclei in mutant brains (figure 1a–d). However, in 7-day old $l(2)gl^4$ larval brain ganglia the number of nuclei that were replicating was considerably higher than in wild type or $l(2)gl^4/SM5$ sibs and with increasing age of $l(2)gl^4$ larvae, the number of replicating nuclei continued to increase further (figure 1e–g).

In the 4- and 5-day old wild type larvae (as also in $l(2)gl^4$ *or*$/SM5$ heterozygotes), the replicating ganglion mother cells (GMC) formed a 2–3 cell thick concentric band in both optic lobes of brain ganglia (figure 1a, c). As the larvae grew older, this intensely fluorescing concentric band on each side became much wider (8–10 cell thick, see figure 1e). The proliferation centres of neuroblasts were seen at dorso-lateral positions of the brain. In addition to the proliferation centres of neuroblasts on dorso-lateral positions and the concentric bands of GMCs in optic lobes, BrdU-incorporation was also seen in some nuclei in medial parts of cerebral ganglia and in ventral ganglion. The spatial distribution of these nuclei, however, did not show any distinct pattern. The number of replicating nuclei in these other regions declined with larval growth (figures 1a, c and e).

In contrast to the wild type pattern, the replicating GMCs in the $l(2)gl^4$ brain ganglia were not arranged in the characteristic concentric bands in optic lobes. Thus although morphologically the brain ganglia of 4/5-day old $l(2)gl^4$ larvae appeared similar to those in wild type larvae of the same age, the distribution of replicating nuclei was strikingly different (see figure 1a–d): the fewer replicating nuclei in the mutant brain were distributed rather uniformly over the dorso-lateral aspects of brain ganglia and the ventral ganglion (figure 1b, d). By day 7, the number of BrdU-labelled nuclei in $l(2)gl^4$ brain was very high but except for a faint suggestion of a concentric band of fluorescence in the optic lobe region, the large number of replicating nuclei did not show a well-defined spatial pattern (figure 1f). From day 8 onwards, the BrdU-labelled nuclei were seen all over the brain without any apparent order. As mentioned earlier, the brain also lost its characteristic shape as the tumourous growth continued (figure 1g).

The stereotyped pattern of divisions of neuroblasts and GMCs to produce neurons in insects leads to formation of clusters of replicating cells in which a large neuroblast cell is associated with smaller GMCs, pre-ganglion and ganglion cells (which ultimately give rise to neurons). These clusters can be easily identified after labelling with BrdU (see Truman and Bate 1988). Clusters of BrdU-labelled

neuroblast, GMCs and ganglion cells were abundantly seen in brain ganglia of 4- to 7-day old wild type larvae (see figure 2). The neuroblast in each of such clusters was differentiated from the GMCs and ganglion cells by its larger size and less intense fluorescence (see figure 2a, c). On the other hand, such discrete clusters were always rare or absent in cerebral ganglia of $l(2)gl^4$ mutants irrespective of larval age (figure 2b, d and e). Some large cells could be seen in $l(2)gl^4$ brains but these were often more intensely fluorescing and were not clustered with smaller cells (figure 2d). However, typical clusters of dividing sister cells could still be seen in the median ventral ganglion of mutant larvae.

4. Discussion

The $l(2)gl^4$ mutation is due to a deletion of about 13·1 kb DNA from the telomeric

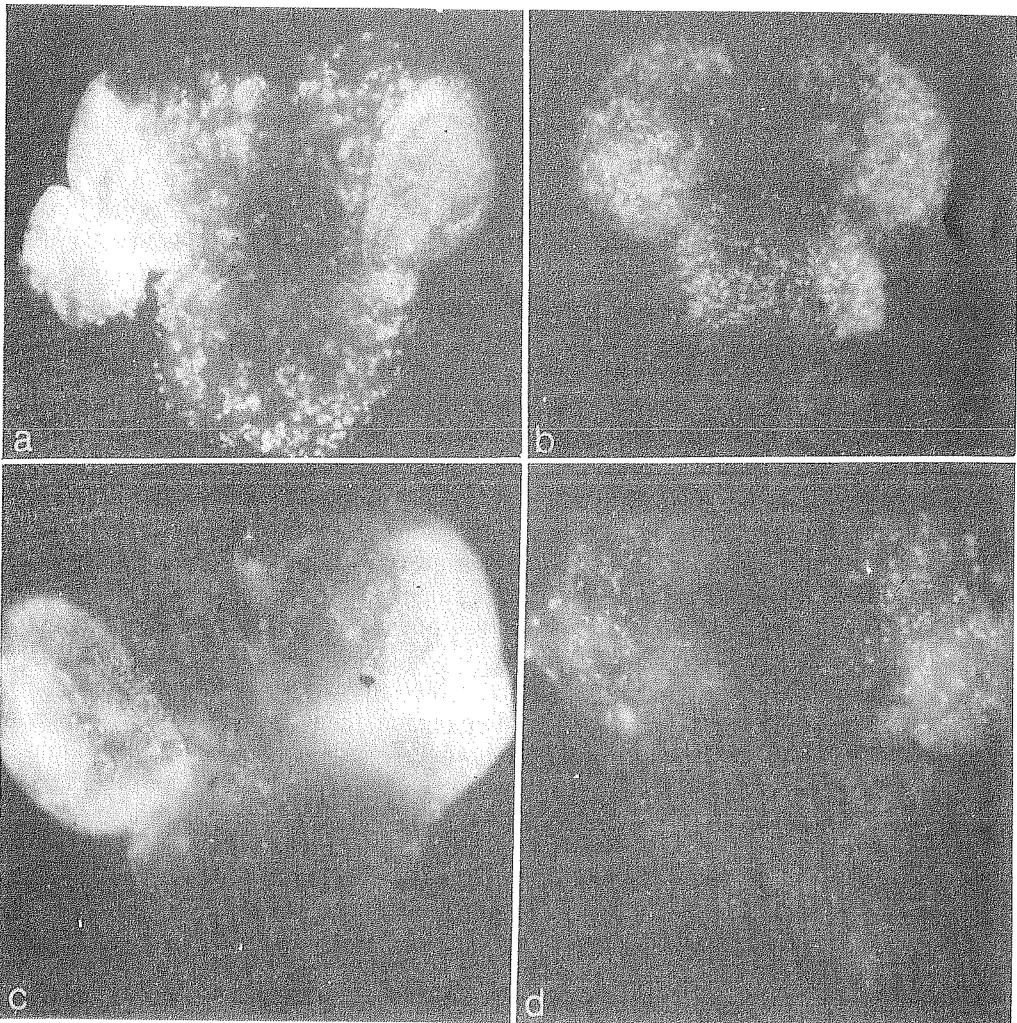

Figure 1. Indirect immunofluorescence showing the spatial distribution of BrdU-labelled nuclei in whole mounts of brain ganglia of wild type (**a**, **c** and **e**) and $l(2)gl^4$ (**b**, **d**, **f** and **g**) larvae on day 4 (a, b), day 5 (c, d), day 7 (e, f) and day 11 (g). (× 300)

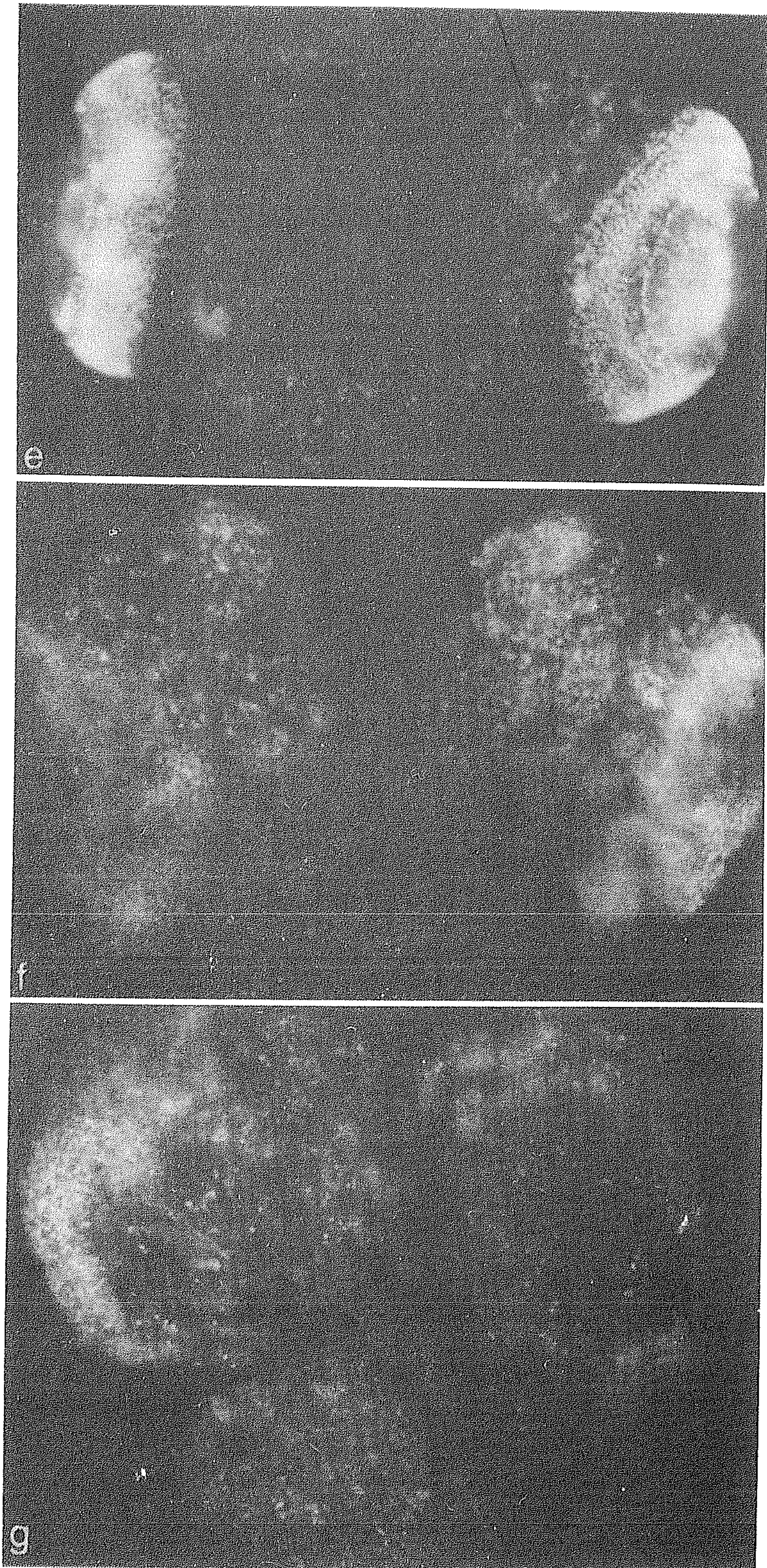

Figure 1. (Caption on facing page)

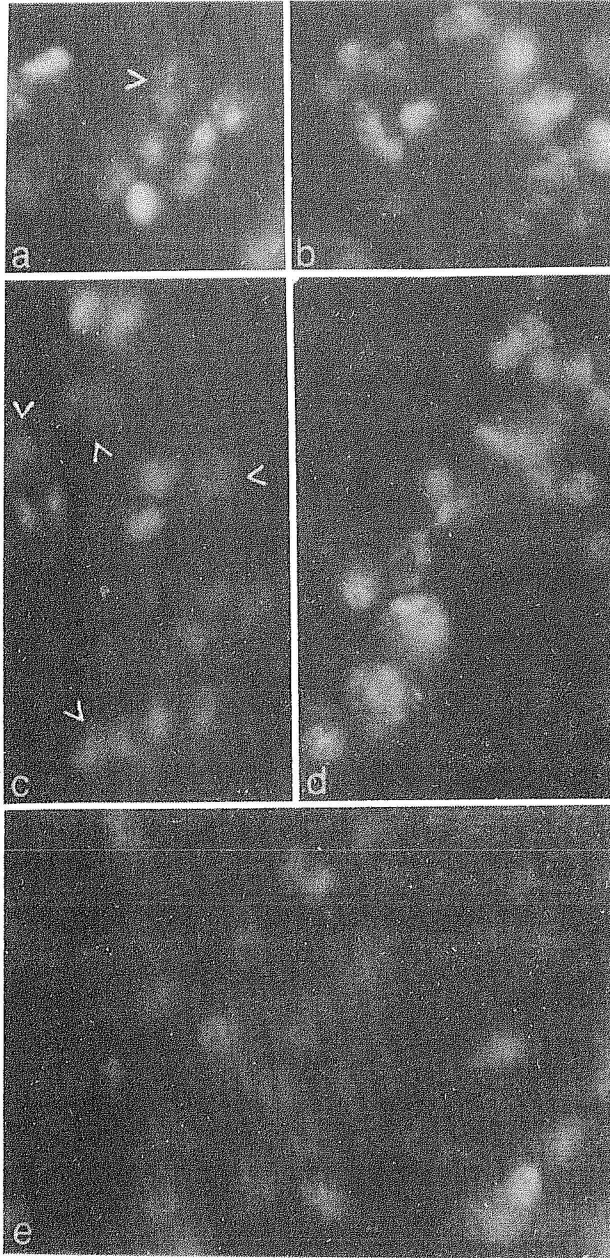

Figure 2. Higher magnification photomicrographs of BrdU-labelled cells from optic lobe areas in whole mounts of brain ganglia from wild type (**a**, **c**) and *l(2) gl*^{4} (**b**, **d**, **e**) larvae on day 4 (a, b), day 5 (c, d) and day 11 (e). Note the discrete clusters of labelled cells in wild type ganglia (a, c): in each cluster, one large and dull fluorescing nucleus (>) is associated with several smaller and brighter fluorescing nuclei. The *l(2) gl*^{4} brain ganglia do not show any such discrete clusters (b, d, e). (× 2300)

region of the left arm of chromosome 2 (bands 21A–B of 2L, Mechler *et al.* 1985; Gateff and Mechler 1989). When homozygous, the mutant condition has profound effect on proliferation and differentiation of cells in the presumptive optic centres of brain and in imaginal discs (Gateff and Schneiderman 1967, 1974). The absence of $l(2)gl^+$ gene activity results in neuroblastoma and benign tumours of imaginal discs. Earlier studies (see review by Gateff and Mechler 1989) revealed that the cortex region of the brain of late $l(2)gl^4$ larvae was considerably enlarged due to many extra divisions of neuroblasts, GMCs and pre-ganglion cells of optic proliferation centres and that these cells failed to differentiate into optic neurons. Our present observations confirmed these results and in addition also revealed two other defects caused by the $l(2)gl^4$ mutation at earlier stages of development. Firstly, in the early 3rd instar larvae, the number of replicating nuclei in $l(2)gl^4$ larval brain ganglia was much less than in wild-type brains. The second and more significant finding of this study was that the pattern of spatial distribution of replicating nuclei was almost completely disrupted in mutant larvae from a very early developmental stage, much before any morphological evidence of the mutant genotype was apparent. This disruption of spatial pattern was evident from the absence of concentric bands of BrdU-labelled fluorescent nuclei in optical regions of the brain ganglia of 4/5-day old mutant larvae and also from a rare occurrence of clusters of neuroblasts, GMCs and pre-ganglion cells. Such clusters of dividing sister cells were absent not only when fewer nuclei were replicating in younger mutant larvae but also when the brain ganglia were hyperactive in cell divisions in older mutant larvae. The presence of clusters of dividing sister cells in median ventral ganglion of $l(2)gl^4$ larvae parallels the earlier observation of Gateff and Schneiderman (1974) that this region does not participate in malignant transformation or tumourigenesis.

Earlier studies of White and Kankel (1978) and of Truman and Bate (1988) suggested a very brief (~ 1 h) cell cycle time for neuroblasts in wild-type brain ganglia while the GMCs and other cells were found to have longer cell cycle durations. In another study (S. Roy and S. C. Lakhotia, unpublished) we compared the mitotic cell cycle in wild-type and $l(2)gl^4$ brain ganglia and found that a sub-population of dividing cells in wild-type brain ganglia which enters mitosis within 15 to 90 min of the S-phase, is largely absent in $l(2)gl^4$ brains. A vital staining of brain ganglia with toluidine blue, which preferentially stains neuroblasts (Truman and Bate 1988), revealed that $l(2)gl^4$ brain ganglia from day 11 larvae had a considerably reduced number of neuroblasts than on day 7 (data not presented). In light of these and our present results, it may be suggested that typical neuroblasts with characteristic rapid and asymmetric division patterns are not present in optic lobes of cerebral ganglia of 3rd instar $l(2)gl^4$ larvae.

Thus the $l(2)gl^+$ gene appears to have a dual regulation of cell division of neuroblasts in brain ganglia: it promotes cell division in brain ganglia in younger stages but limits cell proliferation during the late 3rd instar so that the $l(2)gl^4$ mutant larvae have fewer replicating nuclei in brain ganglia when young but many more when tumourigenesis sets in. A direct count of cells in brain ganglia of $l(2)gl^4$ mutant larvae on different days also reveals that till the early 3rd instar stage, the mutant brains have fewer cells (P. Sinha and A. Mishra, personal communication).

It is known (Gateff and Mechler 1989) that $l(2)gl^+$ gene encodes two major transcripts, one 4·5 kb and the other 6 kb. During early embryogenesis, the 4·5 kb transcript is predominant while during mid- and late-embryogenesis,

the 6·0 kb transcript is seen. A second period of $l(2)gl^+$ gene activity occurs in later larval life (Klämbt and Schmidt 1986; Gateff and Mechler 1989). It has been suggested that the embryonic expression of $l(2)gl^+$ helps in establishing the developmental programmes being decided at that time (Gateff and Mechler 1989). Absence of the $l(2)gl^+$ gene products in $l(2)gl^4$ embryos leads to developmental defects in a variety of larval tissues because cell fates are not properly established. Our present results show that the programmed patterns of asymmetric divisions of neuroblasts and GMCs in specific areas of developing brain are also disrupted due to $l(2)gl^4$ mutation. This was seen as early as day 4, much before the second period of $l(2)gl^+$ gene activity in later larval life (Gateff and Mechler 1989). The altered patterns of cell divisions in $l(2)gl^4$ brain ganglia, therefore, can be attributed to the absence of $l(2)gl^+$ gene products during early embryogenesis. Thus among other things, $l(2)gl^+$ gene activity during embryogenesis is also responsible for defining cell division patterns in optic centres of brain during larval life.

Acknowledgements

This work was supported by a CSIR research fellowship to S.R. and a research grant from the Department of Atomic Energy, Government of India, to S.C.L.

References

Gateff E. and Mechler B. M. 1989 Tumour suppressor genes of *Drosophila melanogaster*. *Oncogenesis* 1: 221–245

Gateff E. and Schneiderman H. A. 1967 Developmental studies of a new mutant of *Drosophila melanogaster*: lethal malignant brain tumour ($l(2)gl^4$). *Am. Zool* 7: 760 (abstract 218)

Gateff E. and Schneiderman H. A. 1974 Developmental capacities of benign and malignant neoplasms of *Drosophila*. *Wilhelm Roux' Arch.* 176: 23–65

Klämbt C. and Schmidt O. 1986 Developmental expression and tissue distribution of *lethal (2) giant larvae* protein of *Drosophila melanogaster*. *EMBO J.* 5: 2955–2961

Lakhotia S. C. and Kumar M. 1980 Replication in *Drosophila* chromosomes. Part VI–Relative heterochromatin content and autonomous endoreplication cycles of hetero- and eu-chromatin in brain cells of *Drosophila nasuta* larvae. *Indian J. Exp. Biol.* 18: 1066–1071

Lindsley D. L. and Grell E. H. 1968 Genetic variations of *Drosophila melanogaster*. Carnegie Inst. Wash. Publ. no. 627

Mechler B. M., McGinnis W. J. and Gehring W. J. 1985 Molecular cloning of *lethal (2) giant larvae*, a recessive oncogene of *Drosophila melanogaster*. *EMBO J.* 4: 1551–1557

Poulson D. F. 1950 Histogenesis, organogenesis and differentiation in the embryo of *Drosophila melanogaster* Meigen. In *Biology of Drosophila* (ed.) M. Demerec (New York: John Wiley & Sons) pp. 209–244

Raman R. and Lakhotia S. C. 1990 Comparative aspects of chromosome replication in *Drosophila* and mammals. In *Trends in chromosome research* (ed.) T. Sharma (New Delhi: Springer–Verlag/Narosa) pp. 69–89

Schubiger M. and Palka J. 1987 Changing spatial patterns of DNA replication in the developing wing of *Drosophila*. *Dev. Biol.* 123: 145–153

Sinha P. and Lakhotia S. C. 1980 Replication in *Drosophila* chromosomes. Part V. Polytene chromosome replication after *in vitro* culture of larval salivary glands. *Indian J. Exp. Biol.* 18: 1059–1065

Truman J. W. and Bate M. 1988 Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Dev. Biol.* 125: 145–157

White K. and Kankel D. R. 1978 Patterns of cell division and cell movement in the formation of the imaginal nervous system in *Drosophila melanogaster*. *Dev. Biol.* 65: 296–321