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Molecular Action of the *l(2)gl* Tumor Suppressor Gene of *Drosophila melanogaster*

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Tumor suppressor genes act as recessive determinants of cancer. These genes contribute to the normal phenotype and are required for regulating cell growth and differentiation during development. Inactivation of tumor suppressor genes leads to an unrestricted pattern of growth in specific cell types. In Drosophila, a series of genes have been identified that cause tissue-specific tumors after mutation. Of these, the lethal(2)giant larvae (*l(2)gl*) gene is the best studied. Homozygous *l(2)gl* mutations cause the development of malignant tumors in the brain and the imaginal discs. Genomic DNA from the *l(2)gl* locus has been cloned, introduced back into the genome of *l(2)gl*-deficient animals, and shown to reinstate normal development. The nucleotide sequence of the *l(2)gl* gene has been determined, as well as the sequences of two classes of transcripts. Analysis of the spatial distribution of both *l(2)gl* transcripts and proteins revealed that during early embryogenesis the *l(2)gl* gene is uniformly expressed in all cells and tissues. In late embryos, the *l(2)gl* expression becomes gradually restricted to tissues presenting no morphological or neoplastic alteration in the mutant animals. Further mosaic experiments revealed that *l(2)gl* gene loss can cause three distinct phenotypes: neoplastic transformation, abnormal differentiation, and normal development. These phenotypes depend upon the extent of gene activity in the stem cells prior to the formation of *l(2)gl*$^-$ clones. These analyses indicate that the critical period for the establishment of tumorigenesis occurs during early embryogenesis at a time when the *l(2)gl* expression is most intense in all cells.

Introduction

About 20 years ago, studies in the field of Drosophila and human genetics revealed that neoplasia may result from recessive mutations in regulatory genes controlling cell growth and differentiation (*1–3*). In *Drosophila melanogaster*, recessive mutations in a series of genes were shown to interrupt the differentiation of certain primordial cells and result in uncontrolled and invasive cell proliferation (*4–6*). As a consequence, the mutant animals form malignant tumors in either the neuroblasts of the optic centers, the imaginal disc cells, or the blood cells. Simultaneous with the appearance and growth of the neoplasms, the development of the mutated animals becomes impaired, and these animals die as larvae or pseudopupae.

In *Drosophila melanogaster*, the best characterized of these loci is the lethal (2) giant larvae (*l(2)gl*) gene. Homozygous mutations of the *l(2)gl* locus are responsible for the malignant transformation of two distinct tissues: the neuroblasts of the presumptive adult optic centers in the larval brain and the imaginal disc cells (*1,7*). In homozygous *l(2)gl*-deficient animals, these abnormalities first become visible in the third larval instar prior to the metamorphosis of the larvae. The tumorous growth produces a complex syndrome characterized by bloating of the larvae, the underdevelopment of the ring gland, and atrophy of the fat bodies. However, the underdevelopment of other tissues such as the gonads and the imaginal cells of the salivary glands occurs before the outgrowth of the tumors and can thus be directly attributed to the absence of *l(2)gl* gene activity in these tissues (*8*).

Using techniques of molecular biology, we have

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cloned the *l(2)gl* gene (*9*) and subjected it to molecular analysis (*10*). This approach has unequivocally demonstrated that the tumorous phenotype results from a lack of gene function. Furthermore, we were able to prevent tumorigenesis by reintegrating a normal copy of this gene into the genome of *l(2)gl*-deficient animals (*10,11*). Such successful gene therapy indicates that the *l(2)gl* gene appears to have the characteristics of a tumor suppressor or antioncogene similar to those recently described in humans (*12–15*).

The molecular analysis of the *l(2)gl* gene, located at the cytological band 21A on the left arm of chromosome 2 (*9,16*), has provided insights into both the biochemical nature of the gene and its mode of action during the development (*9–11*) but has not yet given any convincing clues to any particular function. Elucidation of this function requires information on the expression of the *l(2)gl* gene, in particular on the spatio-temporal pattern of expression of the *l(2)gl* transcripts and *1(2)gl* proteins, on the intracellular localization of the *l(2)gl* proteins and on the critical periods of gene expression that prevent the formation of tumors or any other pathological manifestations. The relevance of this information should then be assessed by introducing specifically modified *l(2)gl* gene constructs that should allow us to determine the spatial and temporal limits of gene action as well as to define the functional domains of the *l(2)gl* gene and its products. In this article, we present our recent progress in the analysis of *l(2)gl* gene expression and the preliminary results that we have obtained by reverse genetics to assess whether the implications of these analyses are relevant to tumorigenesis.

Results

Spatio-temporal Pattern of *l(2)gl* Expression

Northern blot analysis has revealed that the *l(2)gl* gene displays developmentally biphasic expression with an intense period during early embryogenesis and a second period during the larval-pupal transition phase (*9*).

The availability of molecular probes for the *l(2)gl* transcripts and antibodies for the *l(2)gl* proteins has allowed us to investigate the spatial limits of *l(2)gl* expression during embryogenesis. As shown in Figure 1, the *l(2)gl* transcripts are first detected at the end of the syncytial blastoderm stage over all peripheral nuclei. At the cellular blastoderm stage, the *l(2)gl* expression becomes stronger and is found over the cytoplasm in all cells. This expression then remains uniform and relatively intense over all embryonic cells during the gastrulation and germ-band extension stages until about 8.5 hr of embryonic development.

At the time of the dorsal closure (about 10 hr of development), the expression of *l(2)gl* gradually becomes restricted to the epithelial cells of the midgut and the salivary glands, where it persists until the end of embryogenesis and disappears in all other tissues.

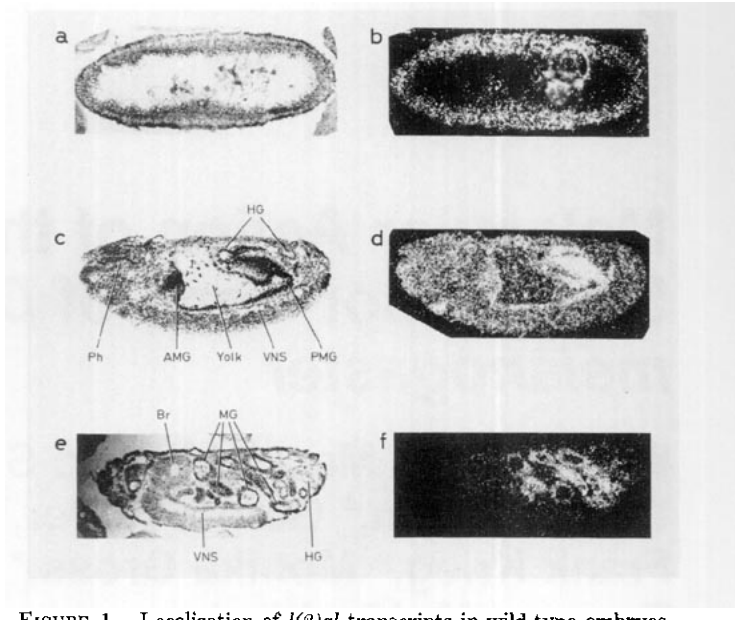

FIGURE 1. Localization of *l(2)gl* transcripts in wild-type embryos. All sections are sagittal and oriented so that anterior is to the left and dorsal up. Localization of *l(2)gl* transcripts in (*a,b*) 2.5-hr (*c,d*) 10.5-hr, and (*e,f*) 18- to 20-hr embryos. (*b,d,* and *f*) Represent dark field photomicrographs of the same sections as in (*a,c,* and *e*), respectively. AMG, anterior midgut rudiment; Br, brain; HG, hindgut; MG, midgut; Ph, pharynx; PMG, posterior midgut rudiment; VNS, ventral nervous system. From the formation of the syncytial blastoderm until germ band retraction, the *l(2)gl* expression is uniformly observed in all embryonic cells and then becomes gradually restricted to the midgut and the salivary glands.

Immunostaining analysis has revealed that the expression pattern of *l(2)gl* proteins follows generally the pattern of *l(2)gl* transcription (Fig. 2). During early embryogenesis, a general and uniform staining is observed over all embryonic cells. In later embryonic stages, the *l(2)gl* proteins are detected in the midgut and the salivary gland epithelia and, in addition, in the axon projections forming the neuropile of the central nervous system. The accumulation of *l(2)gl* proteins in the axons indicate that the *l(2)gl* protein turnover and/or translational efficiency of the *l(2)gl* transcripts in the neuroblasts may be different from that in other embryonic tissues.

Intracellular Localization of the *l(2)gl* Proteins

At the cellular level, the *l(2)gl* proteins appear to be present both in the cytoplasm and bound to the plasma membrane during the early embryonic stages, whereas in later stages the *l(2)gl* proteins appear to be associated essentially with the cell periphery. In particular, the *l(2)gl* proteins present in the polyhedral cells of the midgut epithelium are restricted to domains of the plasma membrane facing contiguous cells and are totally absent from the basal and apical membranes of the epithelial cells.

Although no suggestion of a direct membrane asso-

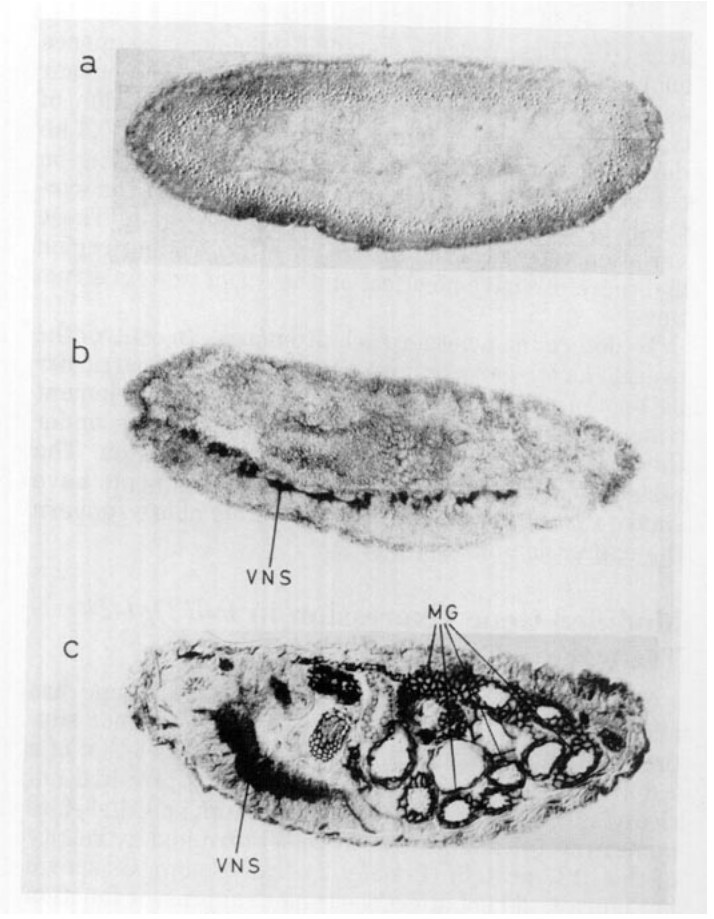

FIGURE 2. Expression of *l(2)gl* proteins in wild-type embryos. All sections are sagittal and oriented so that anterior is to the left and dorsal up. The sections were stained with rabbit anti-*l(2)gl* antibodies. Localization of *l(2)gl* proteins in (*a*) 2.5-hr, (*b*) 12-hr, and (*c*) 18- to 20-hr embryos. The expression of the *l(2)gl* proteins coincides generally with the pattern of *l(2)gl* transcription. The accumulation of *l(2)gl* proteins in the axon projections of the ventral nervous systems (VNS) indicate that the *l(2)gl* protein turnover and translational efficiency in the neuroblasts may be different from that in the other embryonic tissues. In late embryos, the *l(2)gl* proteins present in the polyhedral cells of the midgut (MG) epithelium are associated with the plasma membranes facing contiguous cells and are completely absent from the basal and apical membranes of these cells.

ciation of the *l(2)gl* proteins can be found in the putative *l(2)gl* amino acid sequence, such as an N-terminal leader peptide or a highly hydrophobic transmembrane domain (*10*), we can show by using cell-fractionation procedures that the p127 *l(2)gl* protein is tightly associated with membranes (data not shown). However, the nature of this binding is not yet known.

Critical Periods for Establishment of *l(2)gl* Neoplasia

Previous analysis has revealed that the *l(2)gl* gene is essentially active during embryogenesis and during late third larval instar (*9*). These two phases of *l(2)gl* gene expression correspond to the two major periods of cell proliferation during Drosophila development. It is tempting to attribute this correlation to a direct repression of cell proliferation or an indirect repression caused by induced differentiation that limits the mitotic potential of the cells undergoing this process. However, this hypothesis does not take into consideration the fact that the *l(2)gl*-deficient embryos are able to complete their embryogenesis normally. No apparent morphological or histological differences can be detected in *l(2)gl*-deficient embryos, although the *l(2)gl* embryonic cells have already acquired tumorigenic potential, as shown by transplantation of *l(2)gl* embryonic cells into the abdomen of adult hosts (*7*). The tumorigenic commitment of embryonic cells suggests that the critical period for establishment of neoplasia takes place during early embryonic development, when intense *l(2)gl* gene activity occurs.

A functional assay is required to determine the respective contribution of each period of *l(2)gl* expression. Such an analysis can be performed by means of inducing the development of $l(2)gl^-$ clones in otherwise heterozygous animals and by studying the fate of the clones during development. For this purpose, we have taken advantage of the availability of a functional $l(2)gl^+$ gene associated with the X chromosome (*11*). Using crossing over, we have introduced the gene into a ring X chromosome that is preferentially lost during the first cleavage divisions of the early embryo but that can instead be lost during subsequent mitoses, albeit less frequently. With appropriate genetic markers, we have been able to analyze the fate of $l(2)gl^-$ clones and to correlate the different *l(2)gl* phenotypes with the development phase when the $l(2)gl^+$ chromosomal loss has taken place.

Analysis of the *l(2)gl* mosaic animals indicates that the neoplastic growth takes place in clones of cells that have lost the *l(2)gl* gene in the preblastoderm syncytial embryos prior to any expression of the *l(2)gl* gene (Fig. 3). Clones that are produced at embryonic stages during the first period of *l(2)gl* expression do not display the neoplastic phenotype but are unable to complete differentiation. Finally, when the clones arise during the larval stages, the *l(2)gl*-deficient cells show a nearly nor-

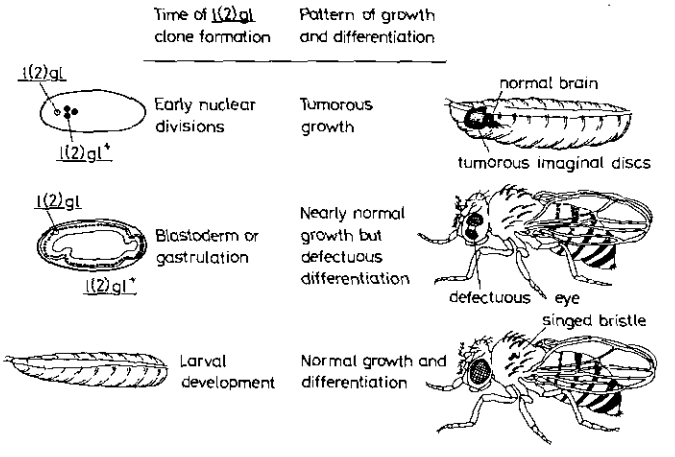

FIGURE 3. Abnormalities produced by $l(2)gl^-$ clones generated at different periods of Drosophila development.

mal or normal development. With the assumption that the *l(2)gl* activity is cell autonomous, these data suggest strongly that the critical period for the establishment of *l(2)gl* neoplasia is during early embryogenesis.

Dissection of the *l(2)gl* Gene

Gene dissection should permit a more precise definition of the function of the *l(2)gl* gene and a better appreciation of its role in causing malignancies and other developmental abnormalities. The cloning of the *l(2)gl* gene allows us to deliberately modify its structure and to test the functional consequences of these structural changes by using reverse genetics.

Previous gene transfer experiments have shown that tumorigenesis can be prevented by introducing a normal copy of the *l(2)gl*$^+$ gene (Fig. 4), which encompasses a 13.1-kb DNA segment, into the germ line of *l(2)gl*-deficient animals (*10,11*). Further experiments with transposons containing resected *l(2)gl* gene segments have allowed us to delimit more precisely the essential domains of the *l(2)gl* gene.

Upstream Regulatory Elements in the *l(2)gl* Gene

The nucleotide sequence analysis has revealed the presence of two 2,8-kb repeats in the 5′ region of the gene (*10*). These repeats share 96% homology. Sequence analysis of a series of cDNAs, S1 mapping, and primer extension experiments have shown that initiation of transcription can occur in both repeats, at about 0.4 kb downstream from their proximal limits. Depending on the splicing pattern, this duplication leads to the synthesis of transcripts that differ in the 5′ region. These variations are localized upstream of the coding region and therefore have no effect on the *l(2)gl* protein structure.

To determine whether each promoter can control the overall *l(2)gl* gene expression or directs a particular pattern of expression, we have constructed P-element transposons (Fig. 4) in which the *l(2)gl* gene is under the control of one or the other promoter region. The results of P-mediated gene transfer experiments have shown that each promoter region is fully able to govern the expression of *l(2)gl* gene.

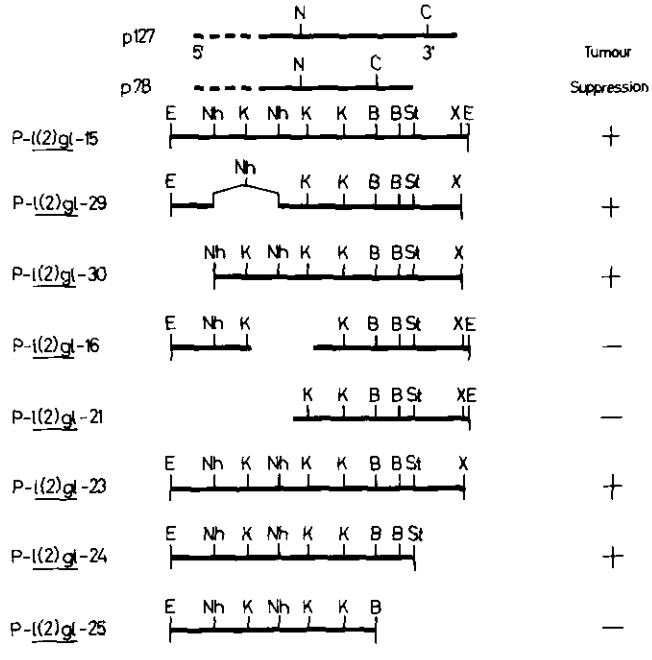

FIGURE 4. Diagrams of P-*l(2)gl* transposons. Above the *l(2)gl* genomic sequence is shown the extent of the two transcripts (including the introns) that encode the p127 and p78 proteins, respectively. The dashed lines represent the transcripts initiated in the first repeat. The position of the initiation and termination codons are indicated by N and C, respectively. On the right are the functional capacities of the P-*l(2)gl* transposons to complement *l(2)gl* deficiencies with respect to tumor suppression. Restriction sites: B, BamH1, E, EcoR1; K, Kpn1; Nh, Nhe1; S, Sal1; St, Stu1; X, Xba1.

Modified Gene Expression in P-*l(2)gl*-24 Transgenic Embryos

Truncation of the 3′ region of the *l(2)gl* gene has allowed us to delineate more precisely the tumor suppressor domain within the *l(2)gl* protein. Deletion of a 2.6-kb DNA segment removing the 1803 terminal nucleotides of the largest *l(2)gl* transcripts and therefore truncating 141 amino acids at the C-terminal extremity of the p127 protein (P-*l(2)gl*-24 transposon), as shown in Figure 4, did not affect the tumor suppressor function of the *l(2)gl* gene (*10*). Immunoblot analysis of the proteins synthesized by P-*l(2)gl*-24 embryos has shown that the largest *l(2)gl* protein is expressed in a shortened form. The apparent molecular weight reduction corresponds to a truncation of the 141 C-terminal amino acids of the *l(2)gl* and their replacement by a sequence of 55 amino acids presumably provided by the read-through into the flanking transposon sequence.

Recent immunostaining analysis of the distribution and localization of the *l(2)gl* proteins has revealed that the *l(2)gl* proteins are no longer associated with the plasma membranes but are found diffused in the cytoplasm of early embryonic cells. These results suggest that the membrane localization of the *l(2)gl* proteins does not necessarily play an important role in the regulation of cell fate because the absence of plasma membrane association of the *l(2)gl* proteins in P-*l(2)gl*-24 embryos does not lead to tumorigenesis. Furthermore, these results suggest that the tumor suppressor function is only required transiently during early embryogenesis.

Conclusions and Prospects

The isolation of the *l(2)gl* gene leaves a number of unresolved questions and paradoxes. Although the gene is expressed in a wide variety of tissues, its inactivation leads only to a small range of tumors. Other tissues are also affected, but their growth properties are reduced instead of enhanced. Thus, in the absence of *l(2)gl* ac-

tivity, three types of growth can occur in the same organism: normal growth and differentiation of the larval cells; atrophy of some adult cell lineages, such as the germ line and the imaginal cells of the salivary glands; and neoplastic transformation of the imaginal disc cells and the neuroblasts of the presumptive adult optic centers in the larval brain.

Equally perplexing is the delayed appearance of the tumors. The malignant pattern of growth only becomes visible during late larval life, although it can be directly correlated with the absence of gene expression during early embryogenesis. Furthermore, the lack of *l(2)gl* expression has no direct visible effect on the *l(2)gl*-deficient embryos. In these embryos morphogenesis and histogenesis proceed normally. No overgrowth of any tissue can be observed, indicating that the potential tumorigenic cells first follow a normal pattern of development with cessation of cell division during late embryogenesis similar to that in wild-type embryos.

Another paradox consists in the absence of membrane association of the modified p127 *l(2)gl* protein in the P-*l(2)gl*-24 transgenic embryos. These embryos give rise to perfectly viable animals. Thus, the importance of the membrane association of the *l(2)gl* protein in the control of normal development is questionable. Further reverse genetic experiments with specific modifications of the *l(2)gl* gene will allow us to analyze the intracellular site of action of the *l(2)gl* proteins with respect to prevention of tumorigenesis.

How the *l(2)gl* gene products achieve the control of cell growth and cell differentiation remains unclear. It is tempting to speculate that the *l(2)gl* gene products interact with other components of a long signaling chain of molecular events. These components may vary, depending on the cellular context. Further genetic and biochemical studies will reveal the nature of the genes and the gene products interacting with *l(2)gl*. These studies will define the contribution of these genes in the establishment of tumorigenesis.

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