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Proliferation: Tissue-Specific Regulation of Cell Cycle Progression by *string* (*stg*) during *Drosophila* Eye Development

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During *Drosophila* eye development, the posterior-to-anterior movement of the morphogenetic furrow coordinates cell cycle progression with the early events of pattern formation. The cdc25 phosphatase *string* (*stg*) has been proposed to contribute to the synchronization of retinal precursors anterior to the furrow by driving cells in G_2 through mitosis and into a subsequent G_1. Genetic and molecular analysis of *Drop* (*Dr*) mutations suggests that they represent novel *cis*-regulatory alleles of *stg* that inactivate expression in eye. Retinal precursors anterior to the furrow lacking *stg* arrest in G_2 and fail to enter mitosis, while cells within the furrow accumulate high levels of cyclins A and B. Although G_2-arrested cells initiate normal pattern formation, the absence of *stg* results in retinal patterning defects due to the recruitment of extra photoreceptor cells. These results demonstrate a requirement for *stg* in cell cycle regulation and cell fate determination during eye development.

Key Words: **eye development; cell cycle; *string* (*stg*); cdc25; *Drop* (*Dr*); *Drosophila*.**

INTRODUCTION

During the development of a multicellular organism the regulated proliferation of stem cells generates populations of cells that are the precursors of the tissues and organs of the adult. An understanding of the mechanisms that regulate cell cycle progression in response to developmental cues and how proliferation controls are integrated with morphogenic movements, differentiation, and cell death remains important goals. With the exception of specialized cell cycles that occur during early embryogenesis in *Drosophila* and *Xenopus* most somatic cell cycles consist of four phases. Replication of DNA occurs during S phase which is followed by a subsequent M phase (mitosis) in which chromosome segregation and cell division occur. During the two intervening gap phases (G_1, preceding S phase, and G_2, which separates the events of S phase from mitosis) regulatory systems termed checkpoint controls monitor critical events of the previous phase to ensure their fidelity and maintain the temporal order of cell cycle progression (Hartwell and Weinert, 1989). Additional regulatory controls active in G_1 determine the cell's response to extracellular signals that promote or inhibit proliferation and likely coordinate the regulation of cell cycle progression with differentiation (Pardee, 1989).

The cyclin-dependent kinases (cdks) are required for cell cycle progression (Murray and Hunt, 1993) and are the targets of checkpoint controls. Activation of cdks is mediated by their association with different cyclin regulatory subunits (Hunter and Pines, 1991; Sherr, 1994), and by a ubiquitous cdk-activating kinase, CAK (Solomon *et al.*, 1992). Negative regulation of cdk activity occurs by three principal mechanisms; posttranslational modification of the kinase subunit, binding of the cyclin/cdk with inhibitory subunits, and proteolysis of the cyclin (see review of Morgan, 1995). The *wee1*/*mik1* kinase inhibits the activity of cyclin/cdk holoenzyme by phosphorylation of a conserved tyrosine on the kinase (Lundgren *et al.*, 1991; Russell and Nurse, 1987). This inhibitory phosphorylation is reversed by cdc25, a tyrosine phosphatase that is required for entry into mitosis from G_2 (Galaktionov and Beach, 1991; Kumagai and Dunphy, 1992; Russell and Nurse, 1986; Sadhu *et al.*, 1990). Mammalian cdc25 phosphatases have

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also been implicated in the regulation of G_1–S progression in normal cells (Jinno *et al.*, 1994), and are thought to contribute in part to the uncontrolled growth of some tumor cells (Galaktionov *et al.*, 1995, 1996). In addition to a positive regulatory role in promoting mitosis, cdc25 phosphatases are the target of negative regulation. In yeast and vertebrate cells, inactivation of cdc25 phosphatases are required for checkpoint controls that mediate cell cycle arrest in G_2 following irradiation (Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997).

Two cdc25 homologs have been identified in *Drosophila:* the major somatic activity is encoded by the *string* (*stg*) gene (Edgar and O'Farrell, 1989), and a second gene *twine* (*twn*) encodes a germline-specific form (Alphey *et al.*, 1992). Phenotypic analysis of *stg* mutants and misexpression experiments suggests that in the embryo *stg* functions as a mitotic inducer (Edgar and O'Farrell, 1990). Although studies describing the effects of *stg* overexpression in the wing (Johnston and Edgar, 1998; Milan *et al.*, 1996a,b; Neufeld *et al.*, 1998) and other imaginal discs (Kylsten and Saint, 1997) suggest a similar function during postembryonic development, the requirement of *stg* for cell cycle regulation and pattern formation in these tissues has been difficult to address due to the failure of stg^- clones to proliferate. Development of the *Drosophila* eye represents a genetically and molecularly tractable system to investigate developmental mechanisms that regulate cell cycle progression. The adult retina consists of approximately 800 ommatidia each containing eight photoreceptor cell neurons (R-cells) and an invariant number of nonneuronal cell types. During the third larval instar stage differentiation of photoreceptor cells occurs in a columnar epithelium, the eye imaginal disc in the wake of the morphogenetic furrow (Ready *et al.*, 1976). Following the anterior-to-posterior movement of the furrow, photoreceptor cell precursors are recruited into the developing clusters in a stepwise fashion (Thomlinson and Ready, 1987). Immediately posterior to the furrow, recruitment of five neuronal precursors (R2–R5, R8) into a precluster is followed by the synchronous S phase of the intervening cells. The remaining three photoreceptors (R1, R6, and R7) and the nonneuronal complement are subsequently recruited from the progeny of this posterior mitotic wave (Wolff and Ready, 1991).

In the anterior eye imaginal disc, progression of the morphogenetic furrow is marked by a transition from asynchronous cell cycles to arrest in G_1. Synchronization of retinal precursor cell cycles occurs in a three- to four-cell-wide domain immediately anterior to the furrow coincident with the domain of expression of *stg* in the eye disc (Thomas *et al.*, 1994). In this report, we show that *Drop* (*Dr*) mutations are likely *cis*-regulatory alleles of *stg*, as they have defects in the expression of the gene in eye and are associated with restriction fragment length polymorphisms (RFLPs) mapping upstream of the transcription start site. In mutants lacking *stg*, retinal precursor cells anterior to the furrow arrest in G_2, accumulate high levels of mitotic cyclins, and have a reduced mitotic index. We find that G_2-arrested cells initiate pattern formation normally but subsequent defects in ommatidial assembly result in the recruitment of additional photoreceptor cells. Our results demonstrate that G_2/M progression in the developing eye is dependent on *stg*, and that G_1 arrest in the furrow is not essential for the onset of pattern formation.

TABLE 1

Drop (*Dr*) and *string* (*stg*) Mutant Alleles Used in This Study

Allele	Mutagen	Phenotype
Dr^1	X ray	Dominant small eye Recessive lethal (24)
Dr^{Mio}	NMS	Dominant small eye Recessive lethal
Dr^{mr21}	EMS revertant of *Mio*	Recessive lethal
Dr^{fa30}	Spontaneous?	Recessive lethal (38)
Dr^{hwy}	Spontaneous	Viable
stg^{7M53}	EMS	Embryonic lethal (25)
stg^{7B69}	EMS	Embryonic lethal (25)
stg^{X1}	X ray	Recessive lethal
Df(3R)DB1	DEB	Recessive lethal
stg^{RXT13}	X-ray revertant of Dr^1	Recessive lethal (24)
$stg^{\Delta AR2}$	Dysgenic	Lethal, embryo RNA^- (26)

Note. DEB, diepoxybutane; NMS, nitrogen mustard.

MATERIALS AND METHODS

Fly strains and genetics. All Drosophila strains were propagated on standard fly food. *stg* and *Dr* alleles used in this study are listed in Table 1. The viable *Dr* allele *highway* is a spontaneous mutant isolated by Hugo Stocker and Ernst Hafen. stg^{x1} and Df(3R)DB1 were induced in the *cn bw; ri e* strain (isoB) (Dong *et al.*, 1997) and identified by their failure to complement the eye and bristle phenotype of Dr^{hwy}. The dominant alleles Dr^1 and Dr^{Mio} are described (Lindsley and Zimm, 1992; Tearle *et al.*, 1994). Dr^{mr21} is an intragenic revertant of Dr^{Mio}. Revertants were induced with ethyl methanesulfonate (EMS) following Lewis and Bacher (1968) and identified in a genetic screen by suppression of the dominant small eye phenotype. Dr^{L2} was isolated by Ed Lewis and will be described elsewhere. The lethal P-element line FA30 was isolated in a molecular screen for insertions near the PTP99A gene. The FA30 line and Df(3R)R3 are described (Hamilton *et al.*, 1995). Df(3R)KE deletes distal 99A through proximal 99B (K. Zinn, personal communication). Except for Dr^{hwy}, all *Dr* alleles are double mutants in *stg* and a second gene uncovered by the Df(3R)KE. The dominant eye phenotypes of Dr^1 and Dr^{Mio} are due to a gain-of-function mutation in the distal gene. Details of the genetics of *Dr* mutations will be published elsewhere. All other *Drosophila* strains are described on FLYBASE or in Lindsley and Zimm (1992).

Induction of mosaic clones homozygous for a recessive lethal Dr mutation. Eye disc clones homozygous for the Dr^{mr21} allele were produced using the flp-mediated recombination system (Xu and Rubin, 1993). The recessive lethal Dr^{mr21} mutation was recombined onto a third chromosome containing an FRT site at 82B. Females of the genotype *ywf, P[hs:flp; ry*$^+$*]; P[FRT, neo*^{R}*] 82B, P[arm:lacZ, w*$^+$*]* were crossed to males of the genotype *P[FRT,*

neo^R] 82B, Dr^{mr21}/TM6b, Tb Sb. Progeny of this cross were collected after 24–48 h in vials and heat-shocked for 1 h at 37°C. Clones were identified by the absence of β-galactosidase immunoreactivity in eye discs dissected from Tb$^+$ larvae of the genotype *P[FRT, neo^R] 82B, P[arm:lacZ, w$^+$]/P[FRT, neo^R] 82B, Dr^{mr21}*.

Histology and immunocytochemistry. Scanning electron microscopy (SEM) and sectioning of adult heads were performed as described (Renfranz and Benzer, 1989). Eye imaginal disc *in situ* hybridizations were done as described (Cubas *et al.*, 1991) using double-stranded digoxigenin probes prepared by random prime labeling of a 1.3-kb *stg* cDNA. Bromodeoxyuridine (BrdU) labeling of S-phase cells in eye imaginal discs was done *in vivo* by a topical perfusion method (Winberg *et al.*, 1992) or *in vitro* by incubation of dissected eye disc/brain complexes in 1 mM BrdU in *Drosophila* Ringer's. BrdU incorporation was visualized using a mouse monoclonal antibody (Beckton Dickinson, 1/100) and horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Bio-Rad). Mitotic figures were quantified by counting labeled nuclei within the apical focal plane of the eye imaginal disc following immunolabeling using a rabbit polyclonal antibody against phosphorylated histone H3 (Upstate Biotechnology, 1/2500). Immunolocalization of mitotic cyclins using mouse monoclonal antibody against cyclin B (Lehner and O'Farrell, 1990) or cyclin A (Lehner and O'Farrell, 1989) was performed as described (Thomas *et al.*, 1994) and visualized by confocal microscopy using a Zeiss microscope. For eye imaginal disc clones, LacZ-positive cells were labeled with polyclonal rabbit anti-galactosidase antibody diluted 1/2000 (Cappel). *Elav* expression was monitored using a rat anti-Elav monoclonal antibody (1/10) obtained from the Developmental Hybridoma Depository. Atonal protein was localized in eye imaginal discs using a rabbit polyclonal antibody (Jarman *et al.*, 1994) as described (Dokucu *et al.*, 1996). Cobalt sulfide staining of pupal retina was performed as described (Cagan and Ready, 1989).

Molecular genetic mapping of RFLPs associated with Dr mutations. P1 clones containing genomic DNA from the 99A cytogenic region were obtained from the Berkeley Drosophila Genome Project and mapped using standard molecular techniques. Polymorphisms associated with *Dr* mutant alleles were identified by genomic Southern blotting initially using the entire P1 insert as probe, and then subsequently the map position was refined using probes derived from smaller subcloned restriction fragments. Because the parental chromosomes for most of *Dr* alleles are unknown, RFLPs associated with a given mutation were considered significant only if they were apparent using three different restriction enzymes.

Heat-shock rescue. Ectopic expression of *stg* was induced by heat shock from the RK2 insertion line (Edgar and O'Farrell, 1990). Daily 1-h 37°C heat pulses were delivered to cultures of the genotype *w; P[hs:stg, w+]RK2/+;Dr^{hwy}*, and rescue efficiency was assayed by sectioning of adult retina. Four eyes/genotype were sectioned in all cases. Heat-shock regimes of greater frequency were found to result in lethality.

RESULTS

Dominant alleles of the *Dr* locus have a small eye phenotype, are homozygous lethal, and exhibit a semilethal genetic interaction with mutations in *stg.* An additional recessive phenotype in the eye was revealed in somatic mosaics in which retinal clones homozygous for a lethal revertant of *Dr^1* contain supernumerary photoreceptor cells (Tearle *et al.*, 1994). A homozygous viable mutant *highway* (*hwy*), whose phenotype and cytogenetic map position suggested it was a recessive viable *Dr* allele, was identified by Hugo Stocker and Ernst Hafen. Adult *hwy* mutant flies have mildly rough eyes that are slightly smaller than those of wild type (Figs. 1A, 1B) and are missing macrochaetes on the notum. In apical cross sections of the wild-type retina, ommatidia containing the rhabdomeres of seven of the eight photoreceptor cells appear as a precise repeating array (Fig. 1C). In sections of the *hwy* mutant eye, ommatidia with one or more additional photoreceptor cells were also readily detected (Fig. 1D). The extra photoreceptor cells present in the mutant ommatidia consisted of both large (R1–6) and small (R7,8) rhabdomeres, suggesting no obvious bias in cell type.

Genetic Complementation between hwy, Dr, and stg Mutant Alleles

The bristle and eye phenotypes of transheterozygous mutant combinations were scored following inter se crosses between the *hwy* mutant and *Dr* and *stg* alleles (summarized in Table 2). Recessive *Dr* alleles and all *stg* alleles failed to complement the bristle phenotype of *hwy*. In the eye, the supernumerary photoreceptor cell phenotype of *hwy* was not complemented by revertants of *Dr^{Mio}* (*Dr^{mr}*) and a recessive lethal *Dr* allele (*Dr^{fa30}*). Sectioning of retinas from transheterozygous mutants revealed extra photoreceptor cells at a frequency identical to that of *hwy* homozygotes. In complementation tests using *stg* mutants, allele-specific effects in the eye were observed *in trans* to *hwy*. Transcriptional null alleles of *stg* (AR2, RXT13) or cytologically visible chromosome deficiencies that remove the *stg* gene failed to complement the supernumerary photoreceptor cell phenotype of the *hwy* mutant. In contrast, EMS-induced alleles of *stg* (7M, 7B69) complemented the retinal phenotype of *hwy*, as sectioning of eyes of transheterozygous *hwy/stg^{7M}* or *stg^{7B69}* flies showed the wild-type number of photoreceptor cells (data not shown). These genetic data show that three independently isolated *Dr* alleles as well as certain *stg* alleles share a common recessive phenotype resulting in the formation of extra photoreceptor cells in the eye.

Recessive Bristle and Eye Phenotype of Dr Mutations Maps near the stg Gene

Previous genetic analysis of dominant *Dr* mutations suggested that they affected *stg* and defined two distally linked genes (Tearle *et al.*, 1994). We employed cytogenetic mapping to determine if the supernumerary photoreceptor cell phenotype of *Dr* was due to a mutation in *stg* or in the distal genes. Recessive phenotypes associated with *Dr* mutations were mapped relative to two

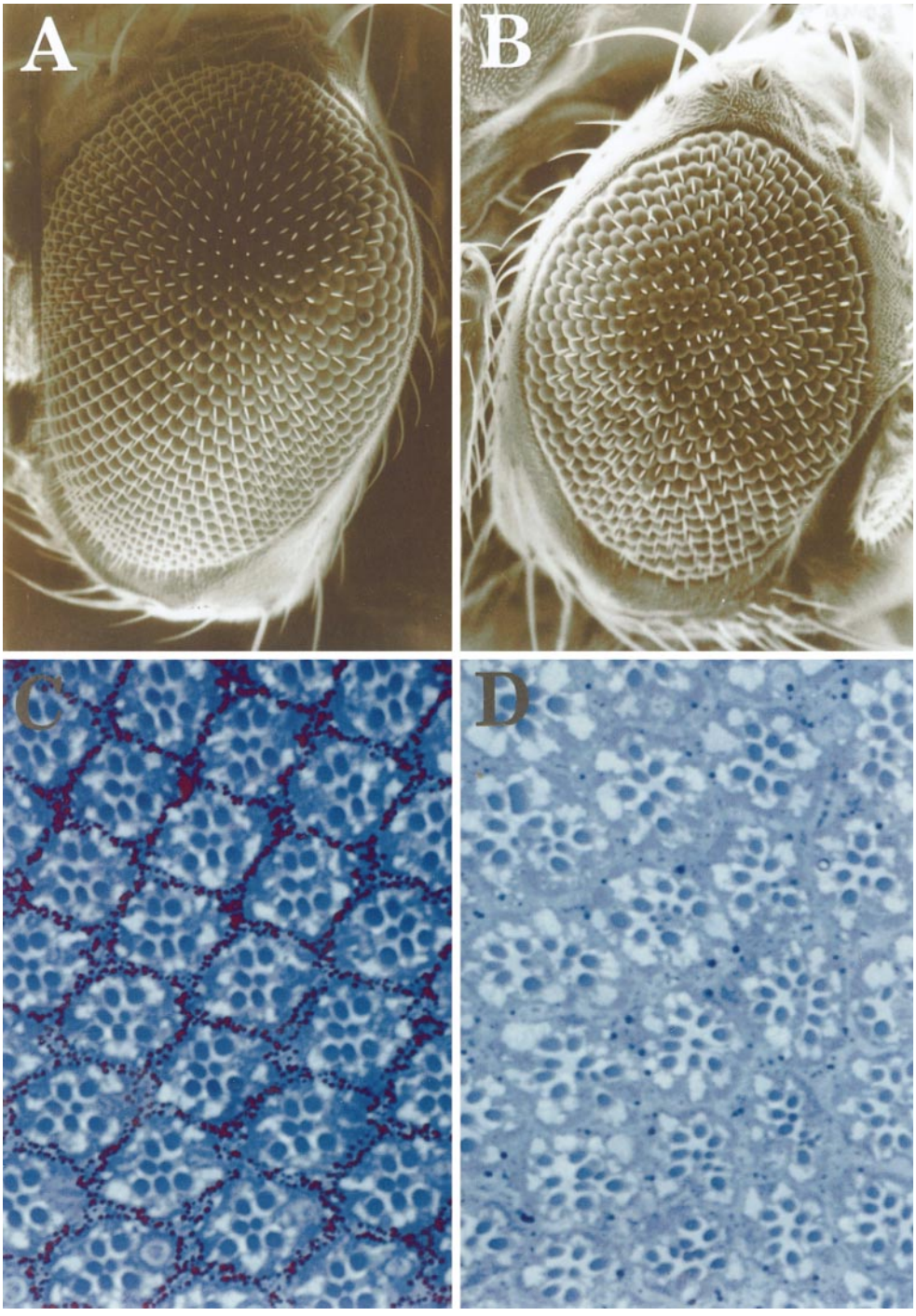


FIG. 1. *highway* is a viable allele of *Dr*. Adult eye phenotype of the Dr^{hwy} mutant. Scanning electron micrograph of wild-type (A) and mutant (B) eyes. Plastic sections (1 μm) of wild-type (C) and *w;* Dr^{hwy} mutant (D) retina. Dr^{hwy} mutant eyes are slightly smaller than wild-type eyes, mildly rough with most ommatidia containing one or more additional photoreceptor cells. Note that the absence of pigment in the section shown in (D) is not due to the Dr^{hwy} mutation.

chromosomal deficiencies [Df(3R)R3 and Df(3R)KE] that separate the 99A region into proximal and distal regions (see Fig. 3 for a description of the deficiency breakpoints). The extra photoreceptor cell phenotype of the *hwy* mutant and the lethality of two recessive alleles, Dr^{mr21} and Dr^{fa30}, were uncovered by the proximal deletion Df(3R)R3. Results

TABLE 2

Genetic Complementation between *Drop* (*Dr*) Alleles and Mutations in the Cell Cycle Regulator *string* (*stg*)

Genotype	Phenotype
Viable alleles	
Dr^{hwy}/Dr^{hwy}	Viable, briste defects,[a] extra R cells
Dr^{hwy}/Dr^{mr} or Dr^{fa30}	Viable, bristle defects, extra R cells
Dr^{hwy}/stg^{point}	Viable, bristle defects
Dr^{hwy}/stg^{-RNA}	Viable, bristle defects, extra R cells
$Dr^{hwy}/Df(3R)R3$	Viable, bristle defects, extra R cells
Lethal alleles	
Dr^{mr} or Dr^{fa30}/stg^{point}	Semilethal, bristle defects
Dr^{mr} or Dr^{fa30}/stg^{-RNA}	Lethal
Dr^{mr} or $Dr^{fa30}/Df(3R)R3$	Lethal

Note. Top: The extra photoreceptor cell phenotype of the *high-way* (*hwy*) mutant is shared by *Dr* alleles and some *stg* alleles. Recessive loss of function *Dr* alleles [intragenic revertants of Dr^{Mio} (Dr^{mr}) and Dr^{fa30}] fail to complement the eye and bristle phenotype of the *hwy* mutant. Complementation of *hwy* with *stg* mutants was allele dependent, with only transcriptional null alleles uncovering the retinal phenotype. The supernumerary photoreceptor cell phenotype associated with *Dr* mutants maps to proximal 99A as $Dr^{hwy}/Df(3R)R3$ heterozygotes have extra R cells. Bottom: Genetic complementation of lethal *Dr* alleles and *stg* mutants. The recessive lethal *Dr* alleles, mr21 and fa30, are poorly viable in combination with *stg* point mutations and have normal eyes and bristle defects. Complementation tests using the same alleles in combination with transcriptional null alleles of *stg* or a chromosomal deletion resulted in lethality. Most *Dr* alleles are double mutants identifying two genes, *stg* and a distal lethal.

[a] Bristle defects refer to missing macrochaetes on the notum.

of genetic complementation and cytogenetic mapping were consistent with the hypothesis that mutations in the *stg* gene contribute in part to some of the phenotypes associated with *Dr* alleles, and this was confirmed by subsequent experiments (see below).

Absence of stg RNA in Eye Precursor Cells Anterior to the Furrow in Dr Mutants

Mutations in *stg* affecting eye development might be expected to alter or eliminate the expression of the gene in retinal precursors. To examine the effects of *Dr* alleles on *stg* gene expression, mutant embryos and eye imaginal discs from third-instar larvae were hybridized with a *stg* cDNA probe. In the wild-type embryo, zygotic *stg* RNA is expressed in a dynamic and complex pattern that highlights mitotic domains (Edgar *et al.*, 1994a; Edgar and O'Farrell, 1989). No obvious defects in the embryonic expression pattern of *stg* were found in mutants homozygous for the dominant allele Dr^{Mio} or for a recessive loss-of-function revertant, Dr^{mr21} (data not shown), suggesting that these alleles do not disrupt embryonic *stg* function.

In the wild-type third-instar larval eye imaginal disc, *stg* RNA is expressed in a stripe of retinal precursor cells immediately adjacent and anterior to the morphogenetic furrow and in additional single cells (Fig. 2A) (Alphey *et al.*, 1992). In contrast, the stripe of *stg* RNA expression ahead of the morphogenetic furrow was absent in eye discs from viable *Dr* mutant larvae or in anterior eye disc clones homozygous for a lethal *Dr* mutation. In the Dr^{hwy} mutant, *stg* RNA is detected only within single cells in the anterior eye disc (Fig. 2B) while expression in the larval brain and in other imaginal discs was unaffected (data not shown), suggesting that *hwy* is an eye-specific allele. Similarly, a defect in the expression of *stg* anterior to the furrow was also observed in eye discs from transheterozygote Dr^{hwy}/Dr^{mr21} and Dr^{hwy}/Dr^{fa30} mutants (data not shown), suggesting that these lethal *Dr* alleles are defective in their ability to express *stg* in the developing eye.

We observed identical effects on *stg* expression in eye disc clones homozygous for a lethal *Dr* allele (Dr^{mr21}). In <5% of the eye discs in which clones were expected to be produced, gaps in the expression of *stg* RNA anterior to the furrow were observed (Fig. 2C, arrow). Defects in the expression of *stg* were never seen in wild-type eye discs.

Molecular Mapping of Lesions Associated with Dr Mutant Alleles

Genetic analysis and *in situ* hybridization experiments suggested that *Dr* mutations contain lesions in the *stg* gene affecting postembryonic expression in the eye. Using probes derived from P1 genomic clones covering >250 kb of DNA, RFLPs associated with three different *Dr* alleles were mapped by Southern blot analysis distal to the *stg* transcription start site (summarized in Fig. 3A). The viable allele, Dr^{hwy}, is associated with an unidentified insertion element, 30 kb upstream from the 5′ end of the *stg* transcript (Fig. 3B). RFLPs associated with the lethal alleles Dr^{1}, Dr^{fa30}, and Dr^{L2} were detected with a more distal genomic probe (Fig. 3C). This probe also identified the distal breakpoint of Df(3R)3450, a chromosomal deletion that uncovers the recessive eye phenotype of *Dr* alleles. As most of the available *Dr* alleles are double mutants, we found additional lesions associated with the dominant eye phenotypes using probes derived from more distal P1 clones corresponding to the cytogenetic interval 99A9,10 (data not shown). The mapping of RFLPs to regions further upstream is consistent with the notion that *Dr* mutations disrupt *cis*-regulatory sequences in the *stg* gene required for expression in eye.

Ectopic Expression of stg Results in the Rescue of the Extra Photoreceptor Cell Defect

To determine whether the extra photoreceptor cell phenotype was due to a failure of *stg* expression in retinal precursor cells, rescue of the eye phenotype was attempted following ectopic *stg* expression. P[w^+, hs:stg]RK2/+; Dr^{hwy} cultures were subjected to daily heat shocks throughout larval and pupal development. In sections of retinas from

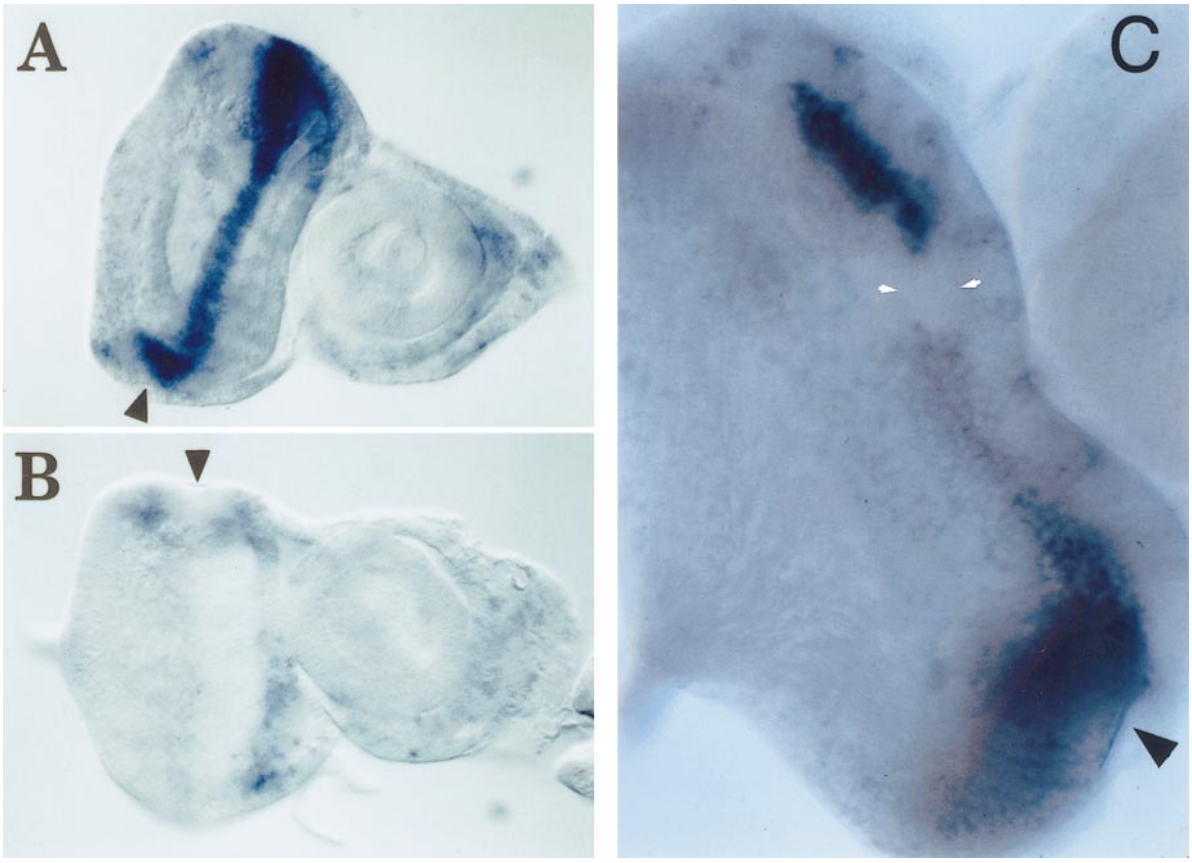


FIG. 2. Recessive Dr mutants fail to express *stg* ahead of the furrow. Expression of *stg* RNAs in retinal precursor cells in wild-type and *Dr* mutant eyes. (A) In the wild-type third-instar eye imaginal disc, *stg* transcripts are localized to a stripe of cells ahead of the morphogenetic furrow (large arrowheads) and in single cells anteriorly. (B) In the Dr^{hwy} mutant eye imaginal disc, the anterior stripe of *stg* expression is absent. Similar defects in the expression pattern of *stg* transcripts were observed in Dr^{hwy}/Dr^{mr21} and Dr^{hwy}/Dr^{fa30} mutant eye discs. (C) In eye disc clones homozygous for the lethal allele, Dr^{mr21} patches of stg^{-} cells (small arrowheads) were observed anterior to the furrow. Anterior to right.

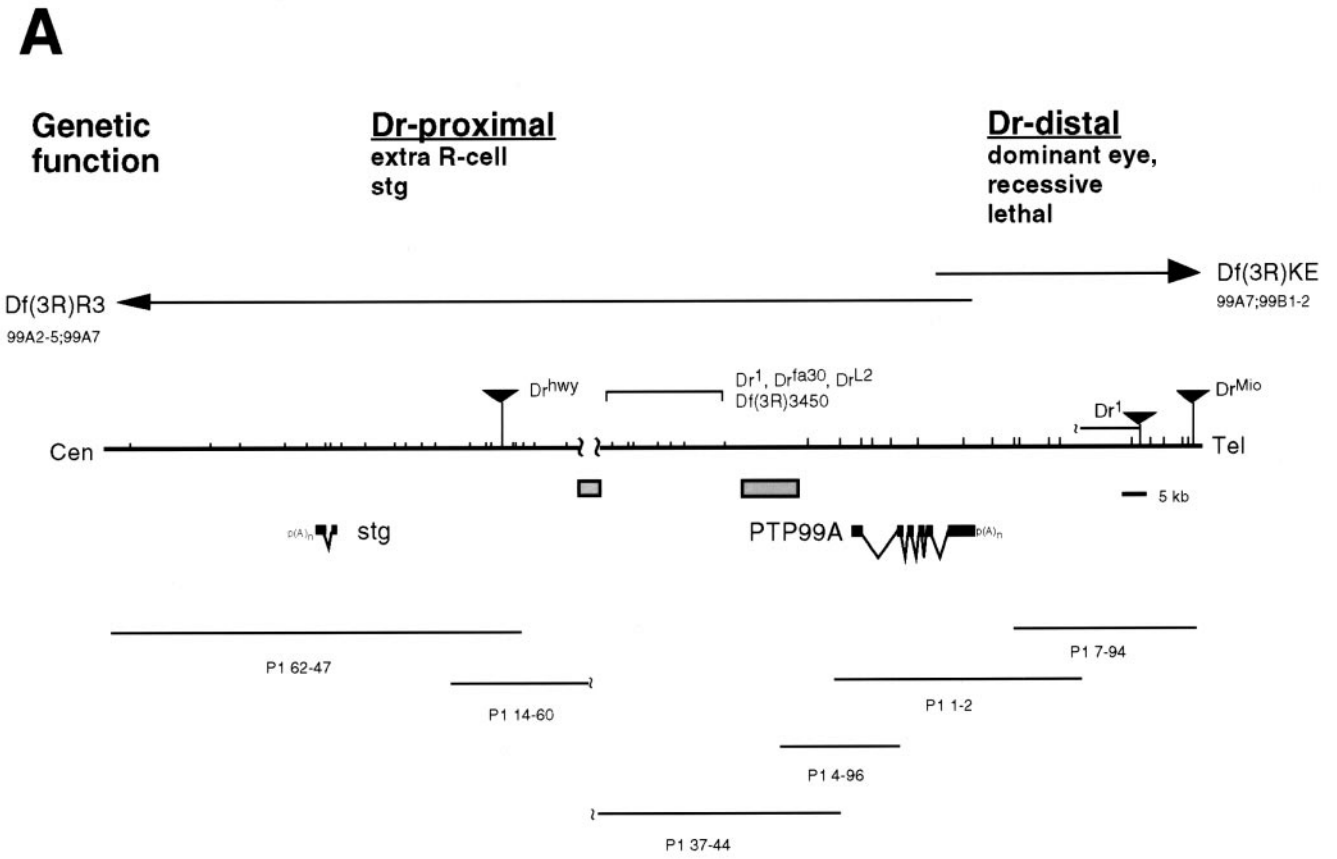
Dr^{hwy} mutant adults with the transgene, 78% of the ommatidia contained the wild-type number of photoreceptor cells (Fig. 4B, $n = 4$). In contrast, sectioning of retina from mutants lacking the transgene, but subjected to the same heat-shock conditions, revealed only 23% wild-type ommatidia (Fig. 4A, $n = 3$). Thus, ectopic expression of *stg* was sufficient to rescue the extra photoreceptor cell phenotype of the Dr^{hwy} mutant, providing additional evidence that *Dr* mutations are allelic to *stg*.

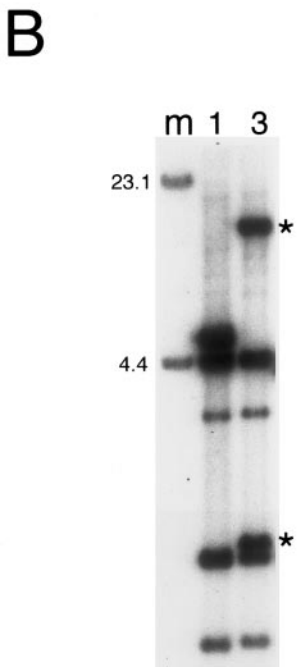
Cell Cycle Defects in Dr Mutants

To determine the function of *stg* during eye formation we analyzed cell cycle progression in third-instar larval eye discs from viable *Dr* mutants or in eye disc clones using cell cycle markers. In the developing eye, S-phase cells were identified using BrdU labeling, G_2 cells by immunostaining with anti-cyclin B monoclonal antibody, and mitotic cells with anti-phosphorylated histone H3 antibody. In the Dr^{hwy} mutant, the pattern and number of S-phase cells in the eye disc were unaffected (Fig. 8), suggesting that *stg* is not required for G_1–S progression.

In the wild-type eye disc, cyclin A (Fig. 5E) and cyclin B protein are uniformly expressed in anterior retinal precursors and abruptly downregulated within the *stg* expression domain and the furrow, coincident with cell cycle synchronization and G_1 arrest (Fig. 5A) (Thomas *et al.*, 1994). In anterior retinal precursors in the Dr^{hwy} mutant eye disc (Fig. 5B) or within anterior Dr^{mr21} eye disc clones spanning the furrow (Figs. 5C, 5D), cyclin B protein is expressed ubiquitously. Similar effects on cyclin A accumulation within the furrow were also observed in Dr^{hwy}/stg^{X1} mutant eye discs (Fig. 5F). Thus, the absence of *stg* ahead of the furrow results in the failure of cyclin A and B downregulation.

The uniform expression of cyclin B within the furrow of *Dr* eye discs suggests that mutant precursor cells arrest their cell cycle in G_2. Consistent with this hypothesis, a decrease in the number of mitotic cells anterior to the





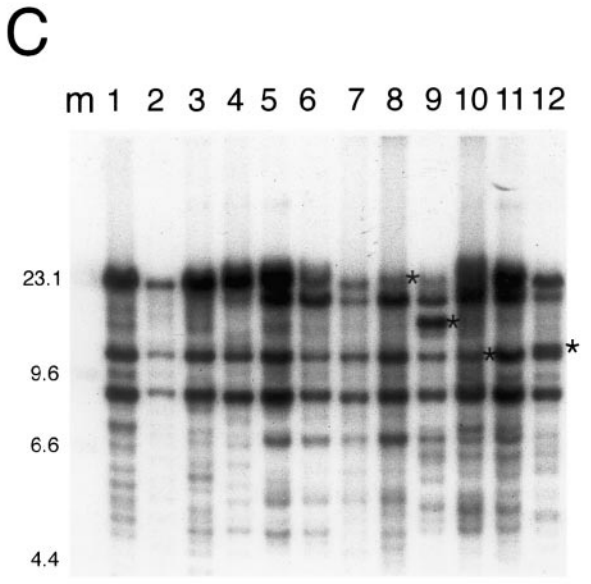


FIG. 3. *Dr* mutations map 5′ to the *stg* coding region and may define *cis*-regulatory sites required for expression in eye. Molecular mapping of RFLPs associated with *Dr* mutant alleles. (A) *Eco*RI restriction map of a >250-kb region of the 99A region on the right arm of the third chromosome. Gaps and regions of uncertainty are indicated (hatched boxes). The position of P1 clones and the location of previously

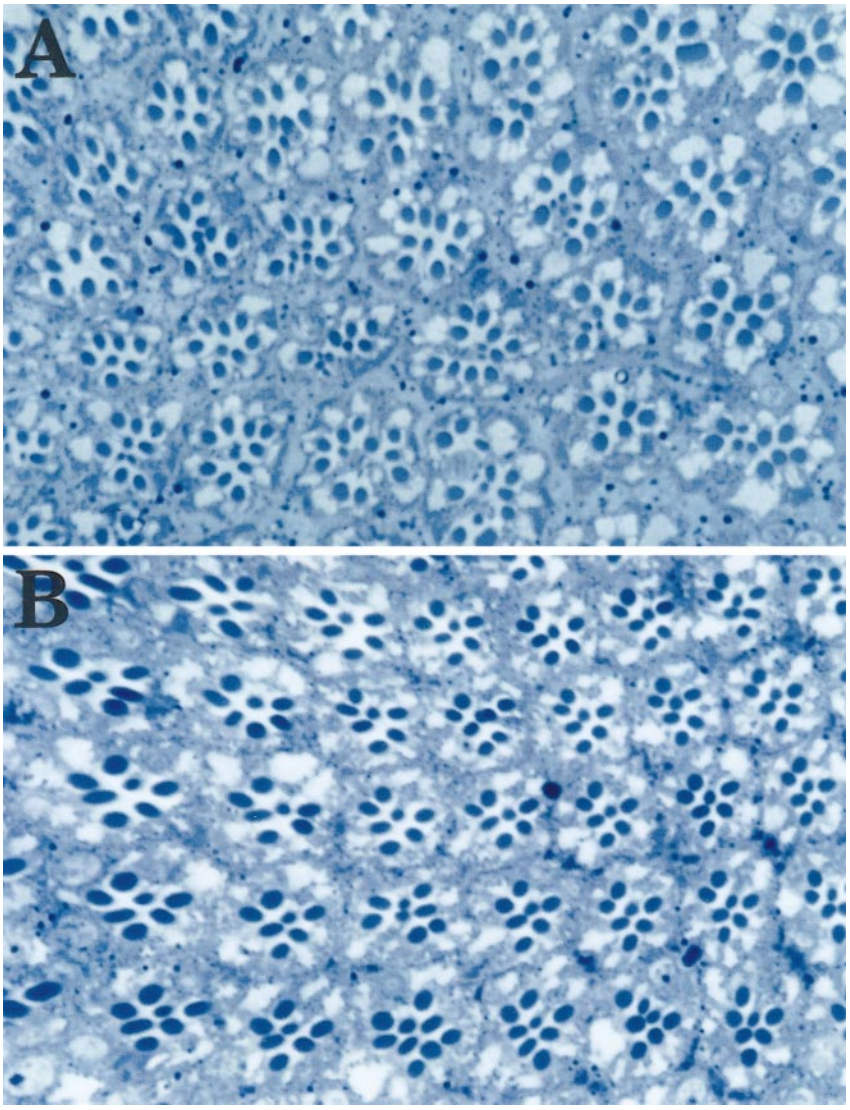
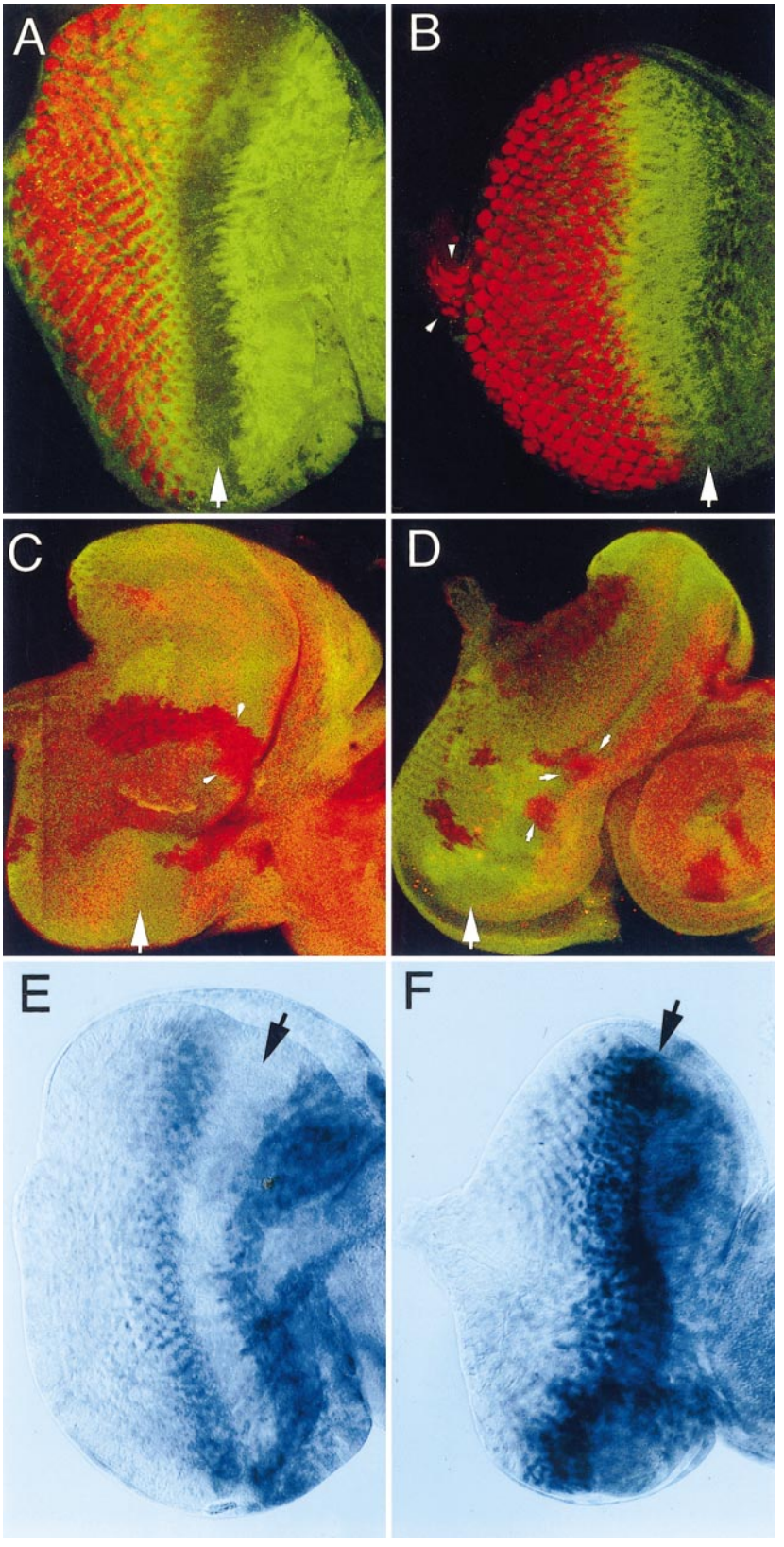


FIG. 4. Rescue of the eye phenotype of the Dr^{hwy} mutant by ectopic expression of *stg.* Apical sections (1 μm) of adult retina from (A) *w;*Dr^{hwy} and (B) *w;P[hs:stg,* w^{+}*]RK2;*Dr^{hwy} derived from cultures subjected to daily heat-shock pulses throughout development. In the Dr^{hwy} mutant eye 78% of the ommatidia contain one or more additional photoreceptor cells (n = 3). In eyes of mutants harboring the *stg* transgene, 23% of the ommatidia contain supernumerary photoreceptor cells (n = 4).

identified transcription units (*stg* and *PTP99A*) are displayed below the map. The two genetic functions affected by *Dr* mutations and the breakpoints of Df(3R)R3 and Df(3R)KE used to map them to proximal and distal 99A are shown above the molecular map. The dominant eye phenotype and recessive lethality of some *Dr* alleles were associated with RFLPs in the distal end of the cloned DNA (data not shown). RFLPs associated with the recessive eye phenotype of *Dr* were mapped to two regions distal to the *stg* transcription start site. (B) The Dr^{hwy} allele is associated with an insertion of unknown DNA. Genomic Southern blot of *Eco*RI-digested fly DNA probed with a 12.0 Xba fragment from P1 14–60. (C) RFLPs associated with the Dr^{1}, Dr^{L2}, and Dr^{fa30} alleles and the distal breakpoint of Df(3R)3450 map within P1 clone 37–44. Genomic Southern blot of *Bgl*II-digested fly DNA probed with the entire insert of P1 clone 37–44 (because of a gap in the cloned DNA, the distance between the RFLPs identified with this probe and the *stg* transcript is not precisely known). The numerous faint hybridizing bands in this experiment are due to a weak repeat present in the P1 clone. DNA samples used are (1) CantonS, (2) OregonR, (3) Dr^{hwy}, (4) isoB [parental strain to stg^{x1} and Df(3R)DB1], (5) stg^{x1}/TM6B, (6) Df(3R)DB1/TM6b, (7) Dr^{Mio}/TM6b, (8) Dr^{L2}/TM6b, (9) Df(3R)3450/TM6b, (10) Dr^{fa30}/TM6b, (11) CB24/TM3 (parental chromosome of Dr^{fa30}), and (12) Dr^{1}/TM3. m, molecular weight markers. Restriction fragments that were altered in size or reduced in the mutants are marked with asterisks. Parental chromosomes for Dr^{Mio}, Dr^{1}, and Dr^{L2} are unknown.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furrow was observed in Dr^{hwy} eye discs. In the wild type, equivalent numbers of mitotic cells are observed in the anterior and posterior regions of the eye disc but are absent within the furrow (Fig. 6A). In contrast, few or no mitotic cells are detected within the *stg* expression domain of the Dr^{hwy} eye disc (Fig. 6B) while mitosis in more anterior cells or behind the furrow was unaffected. Overall, we found a twofold reduction of the number of mitotic cells in the anterior eye disc of the mutant compared with wild type.

Pattern Formation without G_1 Arrest

Retinal precursors cells in the furrow of Dr^{-} eye discs fail to undergo G_1 arrest but instead are blocked in G_2. To investigate the effect of the lack of G_1 arrest on early patterning in the eye, we examined the expression of Atonal, the proneural gene for photoreceptor cell neurons (Jarman *et al.*, 1994). In wild type (Fig. 7A), Atonal is expressed within clusters of cells within the furrow and then becomes restricted to the R8 precursor in the two posterior adjacent rows of cells (Dokucu *et al.*, 1996). In the Dr^{hwy} mutant (Fig. 7B) the pattern of Atonal staining was indistinguishable from that of wild type, suggesting that *stg* (and G_1 arrest) is not required for the onset of pattern formation.

We determined the expression pattern of the neuron-specific marker Elav in wild-type and recessive *Dr* mutant eye discs to examine the events of photoreceptor cell differentiation behind the furrow. In the wild-type eye disc, Elav expression is restricted to the developing photoreceptor cells, commencing behind the furrow initially in a pair of cells in each precluster and then subsequently in all eight photoreceptor cells (Fig. 7C). In the Dr^{hwy} mutant, Elav expression in the developing photoreceptor cells was normal (data not shown); however, in the basal region of the eye disc and within the optic stalk ectopic expression of Elav was observed in the nuclei of glial cells (Fig. 7D).

Using Elav staining it was difficult to identify the supernumerary photoreceptor cells in third-instar larval eye discs from the *Dr* mutants, however, in 24- to 30-h pupal eye discs they were readily apparent (data not shown). To determine the effects of the *Dr* mutants on later patterning events, 48- to 54-h pupal eyes were stained with cobalt sulfide. In the wild-type pupal eye, the outlines of four cone cells and surrounding pigment cells are highlighted (Fig. 7E). In the Dr^{hwy} mutant most of the ommatidia contain one or more additional cone cells (Fig. 7F). In summary, we found that *stg* (and G_1 arrest) is not required for the onset of pattern formation but that it does play a role in later patterning events behind the furrow.

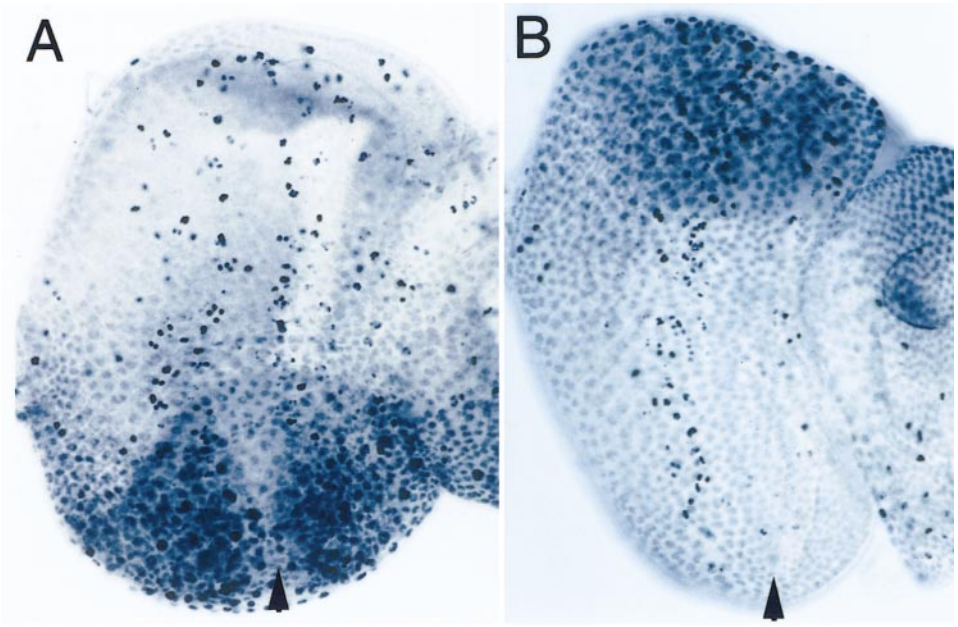
DISCUSSION

In this report we determined the role of *stg* in cell cycle regulation during *Drosophila* eye development. We suggest that *Dr* mutations are novel *cis*-regulatory alleles of *stg.* In recessive *Dr* mutants, retinal precursor cells anterior to the morphogenetic furrow fail to express *stg.* We demonstrated a requirement for *stg* in the transition from G_2 to M in cells ahead of the furrow. Furthermore, we found that the G_1 arrest in the furrow is not a prerequisite for the early events of pattern formation as specification of the R8 photoreceptor cell occurs in G_2-arrested cells in the *Dr* mutant. Finally, our analysis demonstrated a requirement for *stg* in late patterning events behind the morphogenetic furrow.

Drop Mutants are cis-Regulatory Alleles of string

Genetic analysis of *Dr* mutations leads to conflicting interpretations about the question of allelism with *stg.* From the analysis of intragenic revertants of *Dr,* Tearle *et al.* (1994) concluded that the dominant eye phenotype was not due to a gain-of-function mutation in *stg.* However, independently isolated *Dr* alleles all behave as weak hypomorphic *stg* alleles in complementation tests (Tearle *et al.*, 1994; Mozer, unpublished). As we show in the eye, point mutations in *stg* fully complement the supernumerary photoreceptor cell phenotype of *Dr,* while transcriptional null alleles do not. These results could be taken as evidence that the two mutations are not allelic. However, additional experiments do not support this hypothesis. We show that the supernumerary photoreceptor cell phenotype of *Dr* is correlated with defects in *stg* expression in the eye imaginal disc. Furthermore, we find RFLPs in the DNA upstream of the *stg* transcription start site in some *Dr* mutants. Finally, we demonstrate that ectopic expression of *stg* in the eye can rescue the retinal defect of a viable *Dr* allele. These results suggest that *Dr* mutations are alleles of *stg* affecting *cis*-regulatory elements required for the expression in eye. We propose that the lack of an effect in eye in some Dr^{hwy}/stg transheterozygotes is the result of intraallelic complementation.

FIG. 5. Cyclin A and B downregulation in the furrow requires *stg.* Expression of cyclin B in *Dr* mutant eye discs or Dr^{-} eye disc clones. Anterior to right. (A) Wild-type and (B) Dr^{hwy} mutant eye imaginal discs double labeled to reveal the expression of cyclin B (green) and the neuronal marker Elav (red). In wild type, cyclin B is downregulated in the *stg* expression domain and in G_1-arrested cells in the furrow (large arrowhead). In the Dr^{hwy} mutant most cells anterior to the furrow express high levels of cyclin B. (C, D) Eye discs containing Dr^{mr21} homozygous clones, double labeled to reveal cyclin B (red) and LacZ (green). Dr^{-} cells (small arrowheads) in clones spanning the furrow express cyclin B while their wild-type neighbors do not. Cyclin A protein localization in (E) wild-type and Dr^{hwy}/stg^{X1} eye imaginal discs (F) visualized by histochemical staining.



C

	OR			Dr^{hwy}		
	ant	post	p/a	ant	post	p/a
expt1	18	45	2.5	8	22	2.75
	25	48	1.92	-	-	-
expt2	43	46	1.06	21	45	2.14
	33	41	1.24	7	52	7.4
	30	32	1.06	16	47	2.93
	29	30	1.03	14	61	4.36
expt3	31	65	2.1	38	83	2.18
	64	56	0.88	26	62	2.38
	39	52	1.33	35	67	1.91
mean			1.46			3.25

FIG. 6. *stg* contributes to cell cycle synchronization ahead of the furrow by promoting G_2/M progression. Mitosis in *Dr* mutant eye discs. Mitotic figures were identified as apical nuclei labeled with the anti-Phos-histone H3 antibody. (A) In wild type, mitosis occurs in two broad bands ahead and behind the furrow in roughly equivalent numbers of cells. (B) In the Dr^{hwy} mutant, few mitotic cells are detected within the *stg* expression domain, while mitosis in more anterior cells or behind the furrow was unaffected. Anterior to right. (C) Quantitation of mitotic cells in the anterior (ant) and posterior (post) eye disc from wild type and Dr^{hwy} mutants from three separate experiments. The reduction of mitosis anterior to the furrow and the upregulation of cyclin B in the furrow suggest that the absence of *stg* in the Dr mutant results in a G_2 arrest. Anterior to right.

Conserved Function of stg in Cell Cycle Regulation during Eye Development

Experimental studies from yeast to mammals suggest that an evolutionarily conserved function of the cdc25 phosphatases is to activate mitotic cyclin/cdks in G_2 promoting entry into mitosis (Nurse, 1990). In the *Drosophila* embryo, zygotic expression of *stg* is restricted to G_2 cells where it functions as a mitotic inducer (Edgar and O'Farrell, 1989, 1990). We have shown that the absence of the stripe of *stg* expression ahead of the furrow results in the accumulation of the mitotic cyclins and a reduction of mitosis. These observations suggest that mutant precursor cells entering the furrow are arrested in G_2 and demonstrate a requirement for *stg* in G_2/M progression during eye development. The decrease in anterior mitoses in the mutant disc leads to only mild reduction in eye size, presumably because the mutants do not affect the expression of *stg* (and therefore G_2/M progression) in cycling cells in the most anterior part of the disc.

Our experiments do not rule out a function for *stg* in early G_1 cells anterior in the furrow, which express *stg* transcript at high levels (Thomas *et al.*, 1994). In yeast (Amon *et al.*, 1994) and vertebrate cells (Brandeis and Hunt, 1996) negative regulation of G_2 cyclins in G_1 is mediated by proteolysis. The accumulation of the mitotic cyclins within the furrow in *Dr* mutants may reflect a requirement for *stg* in their downregulation in G_1, through a similar pathway targeting them for destruction. In the *Drosophila* embryo, proteolysis plays an important role in the downregulation of the mitotic cyclins following mitosis (Sigrist *et al.*, 1995) and genetic experiments have shown that two structurally related proteins, *Fizzy* (*fzy*) and *Fizzy-related* (*fzr*), are positive regulators of mitotic cyclin degradation (Dawson *et al.*, 1995; Sigrist and Lehner, 1997). Although the role of these proteins in cell cycle regulation in the eye is unknown, it will be of interest to investigate if they are a target of regulation by *stg*.

Dr Mutants Uncouple Cell Cycle Regulation from Early Pattern Formation in the Developing Eye

Classic experiments in tissue culture cells established the importance of regulatory events in early G_1 that coordinate exit from the cell cycle with differentiation (Pardee, 1989). During *Drosophila* eye development the onset of pattern formation is coordinated with G_1 arrest in the eye imaginal disc. The importance of G_1 arrest for retinal cell differentiation was highlighted by the analysis of *roughex* (*rux*) mutants in which precocious entry of retinal precursors into S phase resulted in defects in pattern formation (Thomas *et al.*, 1994). Similarly, abrogation of the G_1 arrest by ectopic expression of cyclin E resulted in S-phase progression in the furrow and subsequent patterning defects (Richardson *et al.*, 1995). We have found that the early events of retinal patterning occur normally in G_2-arrested cells within the furrow of *Dr* mutants. Taken together, these observations suggest that in the developing eye, the initiation of pattern formation can occur from both the G_1 and G_2 phases of the cell cycle, but not S phase. Similarly, cell type determination was relatively unaffected in embryos homozygous for complete loss-of-function *stg* alleles despite a G_2 arrest and the absence of the postblastoderm cell cycles (Edgar and O'Farrell, 1989).

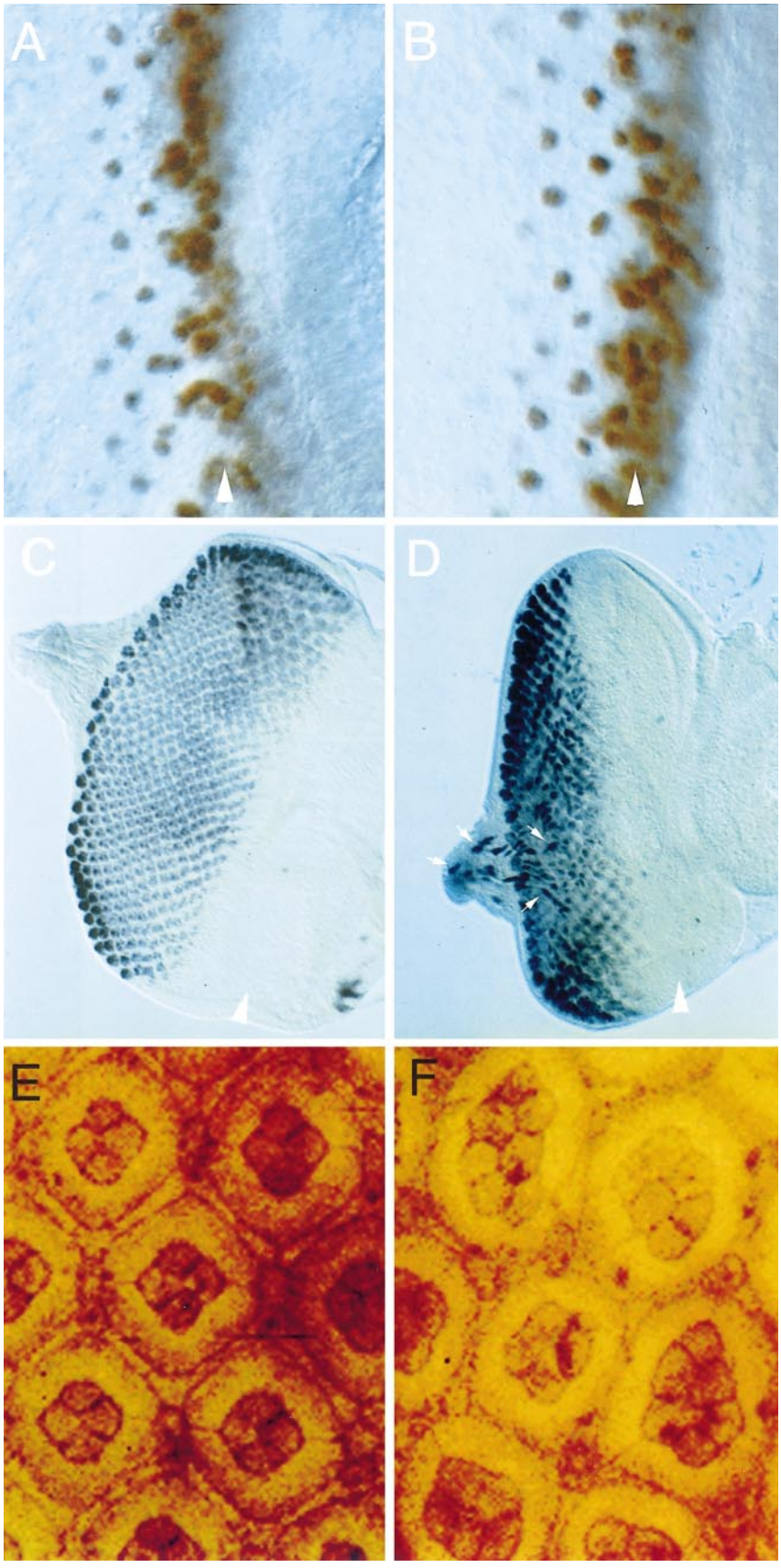
Role of string in Retinal Patterning

In addition to the G_2 arrest phenotype, the absence of the anterior stripe of *stg* in the *Dr* mutant eye disc is also associated with retinal patterning defects resulting in the formation of ommatidia containing supernumerary photoreceptor and cone cells. This may suggest a requirement for mitosis in pattern formation or that *stg* has an additional role in cell fate determination behind the furrow. The recruitment of the normal complement of photoreceptor neurons was unafffected, suggesting that *stg* is not required for their differentiation. We propose that *stg* is required in a subset of retinal precursor cells in the eye disc to antagonize signaling pathways specifying the neuronal cell fate. In the absence of *stg*, these cells are inappropriately recruited to become photoreceptor neurons. Thus, ectopic expression of a neuronal marker (Elav) in retinal glial cells of *Dr* mutants is the result of the failure to downregulate neuronal cell signaling pathways in the eye disc. Cell type specification during *Drosophila* eye development requires a number of ubiquitously expressed molecules that constitute the Ras/Map Kinase signaling cascade (see review by Wassarman *et al.*, 1995). Although a complete analysis of the mechanism of *stg*-mediated patterning in eye is beyond the scope of this report, our observations suggest the possibility that *stg* may contribute cell type specificity through the negative regulation of this pathway.

In the *Drosophila* embryo the cdc25 tyrosine phosphatase encoded by *stg* activates mitotic cdks via the dephosphorylation of cdc2, allowing entry into mitosis from G_2 (Edgar *et al.*, 1994b). Mitotic cdks may also be potential biochemical targets of *stg* that mediate its function in patterning of the eye. Using a gene dosage assay, we tested chromosomal deletions removing the cyclin A and B genes for their ability to modify the eye phenotype of the Dr^{hwy} mutant and found no effect (B. Mozer, unpublished). Evidence for a role of the mitotic cyclins in cell cycle regulation in eye has been well documented (Dong *et al.*, 1997; Thomas *et al.*, 1994, 1997), but their role in patterning is difficult to address. It will be of interest to determine in future experiments what role these molecules play in *stg*-mediated patterning, and to apply genetic approaches to the discovery of additional biochemical targets.

Upstream Regulators of stg in the Developing Eye

Cell cycle regulation and cell fate specification are coordinately regulated during retinal development by the movement of the morphogenetic furrow. Progression of the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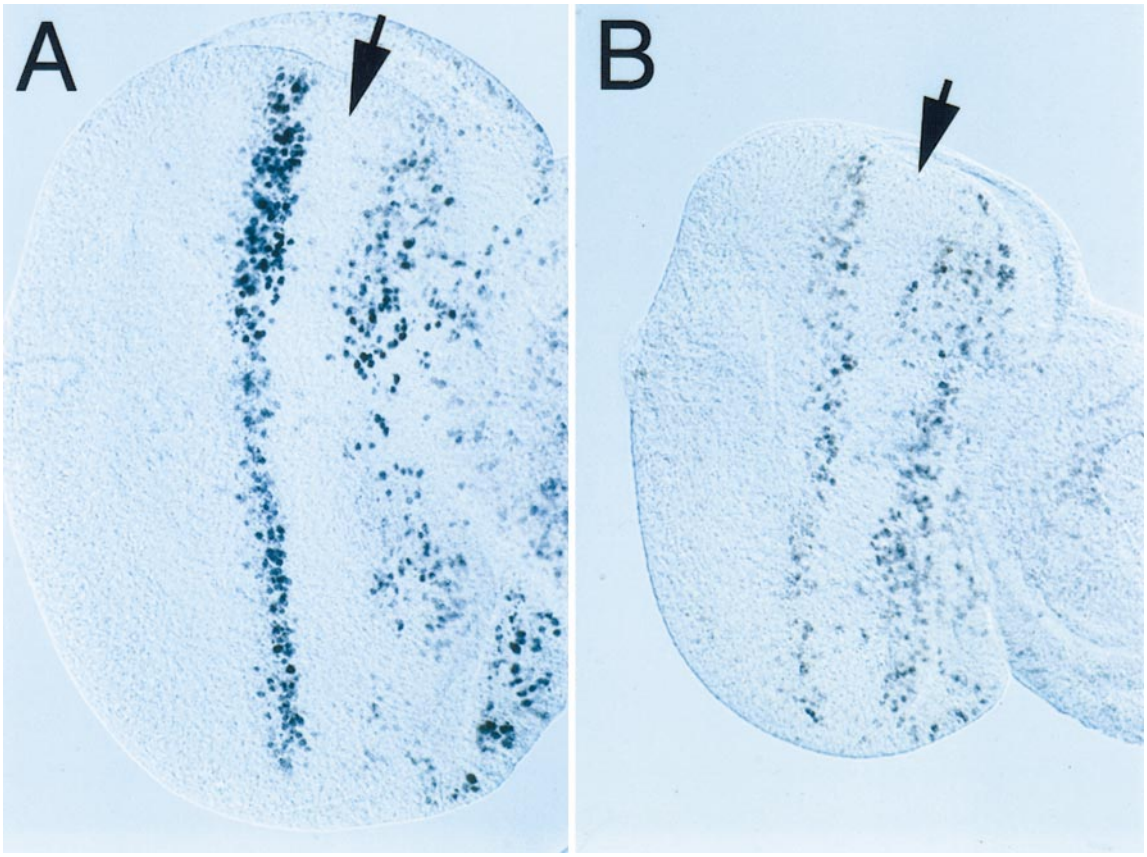

FIG. 8. Supernumerary photoreceptor and cone cells in *Dr* mutant retinas are not the result of additional cell proliferation. S-phase cells revealed by BrdU labeling in wild-type (A) and Dr^{hwy}/stg^{X1} mutant (B) eye imaginal discs. In the wild-type disc, S-phase cells are visible among the asynchronously cycling cells anterior to the furrow and within a stripe behind the furrow, the posterior mitotic wave. No S-phase cells are detected within the furrow. In the *Dr* mutant the pattern and number of S-phase cells were normal. Anterior to right. Arrows indicate the position of the furrow.

furrow is driven by the secreted morphogen *hedgehog* (Ma *et al.*, 1993) which induces the expression of the transforming growth factor-related gene *decapentaplegic (dpp)* within the furrow (Heberlein *et al.*, 1993). Anterior eye disc clones in the furrow that lack a *dpp* receptor accumulate cyclin B protein and initiate mitosis inappropriately (Penton *et al.*, 1997), suggesting that *dpp* signaling regulates G_2/M progression. However, adult eye clones lacking a *dpp* receptor have missing photoreceptor cells (Penton *et al.*, 1997) or no defects (Burke and Basler, 1996), suggesting that cell cycle synchronization by *dpp* is *stg* independent. The activation of *stg* gene expression ahead of the furrow is thought to be

FIG. 7. Early and late patterning in recessive *Dr* mutants. The onset of pattern formation occurs despite the lack of *stg* and G_1 arrest in the furrow. Expression of the proneural gene Atonal in wild-type (A) and Dr^{hwy} mutant (B) eye discs. In wild-type eye imaginal discs, Atonal protein is expressed in clusters in the furrow and then becomes restricted to single cells (the R8 precursor) in several rows of preclusters behind the furrow. The pattern of Atonal protein in the *Dr* mutant is indistinguishable from that in the wild type, suggesting that R8 specification and the early events of retinal patterning can occur during the G_2 stage of the cell cycle. Anterior to right. *Stg* is required for late patterning behind the furrow. Expression of the neural-specific marker Elav in wild-type (C) and Dr^{hwy}/stg^{X1} (D) eye discs; same focal plane (basal) shown for both. In wild type Elav is restricted to the developing photoreceptor cells. In the *Dr* mutant Elav is ectopically expressed in the nuclei (small arrows) of glial cells in the bottom of the disc and in the optic stalk. Anterior to right. Cell profiles of 48- to 54-h-old pupal retina from wild type (E) and Dr^{hwy} mutant (F) highlighted with cobalt sulfide. In the wild type, a reiterated pattern of four cone cells surrounded by two primary pigment cells is visible in each cluster. In the *Dr* mutant most of the ommatidia contain one or more additional cone cells.

the result of *hedgehog* signaling (Heberlein *et al.*, 1995); however, eye disc clones lacking *smoothened* (*smo*), a downstream component of the hedgehog signaling pathway, have no effect on retinal patterning (Strutt and Mlodzik, 1997). Given the observation that viable *Dr* mutants or lethal Dr^{-} adult eye clones have patterning defects, we suggest that in the eye the regulation of *stg* by *hedgehog* is not direct.

In the embryo, activation of *stg* expression is dependent on a number of genes involved in embryonic patterning as well as *cis*-regulatory sequences upstream of the transcription start site (Edgar *et al.*, 1994a; Lehman *et al.*, 1999). Although the genes that mediate activation of *stg* gene expresson in the imaginal discs are unknown, the *Achaete* and *Scute* genes have been implicated in the negative regulation of *stg* expression in the wing disc (Johnston and Edgar, 1998). Molecular genetic mapping of RFLPs associated with *Dr* alleles to a large region (>80 kb) upstream of the *stg* coding region should lead to the identification of the *cis*-regulatory elements in the *stg* promoter that specify expression in the eye. In addition, genetic screens using the eye phenotypes of *Dr* mutants should prove fruitful as a means to identify the *trans*-acting factors that bind to these sites as well as additional molecules that coordinate cell cycle progression and pattern formation during development.

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