An Overexpression Screen in Drosophila for Genes That Restrict Growth or Cell-Cycle Progression in the Developing Eye

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ABSTRACT

We screened for genes that, when overexpressed in the proliferating cells of the eye imaginal disc, result in a reduction in the size of the adult eye. After crossing the collection of 2296 EP lines to the *ey-GAL4* driver, we identified 46 lines, corresponding to insertions in 32 different loci, that elicited a small eye phenotype. These lines were classified further by testing for an effect in postmitotic cells using the *sev-GAL4* driver, by testing for an effect in the wing using *en-GAL4*, and by testing for the ability of overexpression of *cycE* to rescue the small eye phenotype. EP lines identified in the screen encompass known regulators of eye development including *hh* and *dpp*, known genes that have not been studied previously with respect to eye development, as well as 19 novel ORFs. Lines with insertions near INCENP, *elB*, and *CG11518* were characterized in more detail with respect to changes in growth, cell-cycle phasing, and doubling times that were elicited by overexpression. RNAi-induced phenotypes were also analyzed in SL2 cells. Thus overexpression screens can be combined with RNAi experiments to identify and characterize new regulators of growth and cell proliferation.

THE progression of a cell through the cell cycle is subject to a number of controls. Both extracellular and intracellular signals combine to regulate the activity of proteins that directly control cell-cycle progression. Moreover, passage from one cell-cycle state to another is controlled by checkpoints that act to ensure that a cell has completed the requirements of each stage prior to proceeding to the next stage. For example, in response to mitogenic signals, active complexes of cyclins and cyclin-dependent kinases (cdks) are formed and act positively to drive the cell though S phase. If the cell fails to repair DNA damage, negative controls such as cdk inhibitors (CKIs) halt cell-cycle progression. Negative regulators of the cell cycle have been the focus of many studies partly because many of them function as tumor suppressor genes.

In vertebrates, many negative cell-cycle regulators have been identified. These include proteins that directly regulate the cell cycle, such as the retinoblastoma (Rb) protein and its relatives, the CIP/KIP class of cdk inhibitors, as well as proteins that function in signaling pathways that eventually regulate growth or cell-cycle progression such as p53, PTEN, and APC. The Drosophila genome encodes at least two Rb-related genes (Du *et al.* 1996; M. D. Adams *et al.* 2000) and one CKI, *dacapo* (*dap*; de Nooij *et al.* 1996; Lane *et al.* 1996). The *dap* gene encodes a homolog of the p21/p27 family of CKIs. Loss-of-function mutations in either *Rbf* or *dap* do not result in uncontrolled proliferation in the Drosophila embryo. Embryos mutant for *Rbf* fail to maintain a G1 arrest and reenter the cell cycle (Du and Dyson 1999). Cells in embryos mutant for *dap* fail to exit from the cell cycle at precisely the right time. Typically, cells complete an additional cell cycle before becoming quiescent. The relatively subtle phenotypes elicited by mutations in either *Rbf* or *dap* suggest that additional mechanisms may exist to restrict cell proliferation.

We have chosen to use a screen that utilizes gain-of-function mutations to identify genes that restrict growth or cell-cycle progression. Classical genetic screens induce mutations using either X-irradiation or chemical mutagens such as ethyl methanesulfonate. These mutagens mostly generate loss-of-function mutations. Such an approach has led to the discovery of many important genes and pathways that regulate cell proliferation but may also fail to identify genes with subtle loss-of-function phenotypes and genes with redundant functions. In mice, a deletion of the gene encoding the CKI p21 does not result in obvious phenotypic abnormalities; these mice are viable and fertile (Deng *et al.* 1995). However, ectopic expression of p21 results in a cell-cycle arrest in G1 and experiments of this nature have provided important insights into the function of the gene. It has been estimated that >66% of Drosophila genes are phenotypically silent when mutated to a loss of function (Miklos and Rubin 1996) and hence would not be identified in conventional genetic screens. By screening for gene function using forced overexpression in specific tissues, phenotypes may be obtained that might be more recognizable than those elicited with loss-of-function mutations in the same gene.

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To facilitate systematic misexpression screens in Drosophila, Rørth has established a collection of 2300 Drosophila stocks referred to as EP lines (Rørth 1996; Rørth *et al.* 1998). Each EP line carries a *P* element containing GAL4-binding sites and a basal promoter oriented to direct the expression of the genomic sequences downstream of the EP insertion site. It has been demonstrated that Drosophila *P* elements preferentially insert in the 5′-untranslated region (5′-UTR) of genes (Zhang and Spradling 1993). Thus, GAL4 expressed in specific temporal and spatial patterns can be used to drive expression of the gene that is downstream of the EP insertion. We have screened the EP collection using an *eyeless-GAL4* (*ey-GAL4*) driver to detect phenotypes elicited by overexpression of genes in the Drosophila eye. The development of the Drosophila eye has been well characterized and minor defects in the patterning of the eye are easily identified. Moreover, the eye is not required for viability. Using such a screen, we have identified a number of genes that, when overexpressed, reduce the size of the adult eye. Furthermore, we present a detailed analysis of the function of three of these loci.

MATERIALS AND METHODS

Drosophila strains and cultures: Flies were grown on a standard cornmeal medium at 25° unless otherwise specified. The UAS-*p21* and UAS-*dacapo* stocks were generated in our laboratory (J. de Nooij, M.-G. Wang and I. K. Hariharan, unpublished data). The *UAS-Rbf* transgenic line was a gift from N. Dyson. The *ey-GAL4* driver line was a gift from J. Treisman. The EP collection was obtained from the Berkeley Drosophila Genome Project (BDGP; Rørth *et al.* 1998). Individual EP lines were crossed to the *ey-GAL4* driver at 28°. Both *sev-GAL4* (Basler *et al.* 1989; Bowtell *et al.* 1989) and *prd-GAL4* stocks were obtained from the Drosophila stock center at Bloomington, Indiana. The *engrailed-GAL4* line directs expression in the posterior compartment of the wing (Neufeld *et al.* 1998). A recombinant chromosome (II) carrying *ey-GAL4* and UAS-*Cyclin E* (Lane *et al.* 1996) was generated and used to test for suppression of reduced eye size by cyclin E overexpression. The stocks, UAS-*CycD* and *Act5c > CD2 > GAL4* UAS-GFP_{NLS}*S65T* (III), were gifts from B. Edgar. UAS-*p35* (II) was a gift from B. Hay.

***In situ* hybridizations and immunohistochemistry:** cDNA clones of the inner centromeric protein (INCENP; LD34828) and *CG11518* (GH25362) were obtained from Research Genetics (Huntsville, AL). A 482-bp fragment of the predicted third exon of the *elbowB* open reading frame (ORF; *CG4220*) was amplified by PCR and cloned into pBluescript SK+ vector (Promega, Madison, WI). Each linearized cDNA was used as a template for *in vitro* transcription using the digoxigenin RNA labeling kit (Boehringer Mannheim, Indianapolis). RNA *in situs* were performed as described previously (Mlodzik *et al.* 1990). For analysis of the INCENP mutant phenotype, embryo immunostainings were performed as described previously (Patel 1994). Embryos were stained with the YOYO DNA stain (Molecular Probes, Eugene, OR) and anti-actin antibody (ICN Biochemicals) to visualize DNA and cell outlines.

Flow cytometry and cell-cycle analysis: The genotypes used for fluorescence-activated cell sorter (FACS) analysis were as follows: (1) *y w hsFLP/+*; EP2039/+; *Act5c > CD2 > GAL4* UAS-GFP_{NLS}*S65T/+*; (2) *y w* hs*FLP/+*; +; EP1076/*Act5c > CD2 > GAL4* UAS-GFP_{NLS}*S65T*; and (3) *y w* hs*FLP/+*; EP2340/+; *Act5c > CD2 > GAL4* UAS-GFP_{NLS}*S65T/+*. For flow cytometry, larvae were heat-shocked at 72 hr after egg deposition (AED) to induce EP expression. At 96 hr AED, third instar wing imaginal discs were dissected and processed as described (Neufeld *et al.* 1998) and run on a Cytomation MoFlo instrument. The data were analyzed using FlowJo (Tree Star). Measurements of clone size were performed in a p35 background with the following genotypes: (1) *y w hsFLP/+*; EP2039/UAS-*p35*; *Act5c > CD2 > GAL4* UAS-GFP_{NLS}*S65T/ +*; (2) *y w* hs*FLP/+*; UAS-*p35/+*; EP1076/*Act5c > CD2 > GAL4* UAS-GFP_{NLS}*S65T*; and (3) *y w* hs*FLP/+*; EP2340/UAS-*p35*; *Act5c > CD2 > GAL4* UAS-GFP_{NLS}*S65T/+*. Clones were induced at 72 hr AED and fixed at 120 hr AED. Doubling time was determined using the formula (Log 2/Log *N*)hr, where *N* is the median cell number per clone and hr is the age of the clones in hours.

RNA interference and Northern analysis: dsRNA was generated using the Ribomax Large Scale RNA Production-T7 kit (Promega). Drosophila SL2 cells were seeded in a six-well culture dish at a concentration of 1.0×10^6 cells/ml in Schneider's medium supplemented with 10% fetal bovine serum. Cells were washed once with Schneider's medium and then incubated with 50 μg (*elB, noc*) or 25 μg of dsRNA (INCENP) in 1 ml of Schneider's medium. After 30 min, 2 ml of Schneider's medium (with fetal bovine serum) was added. Cell counts were performed each day using a hemocytometer. After 4 days, cells were harvested, fixed, and stained with propidium iodide. FACS analysis was performed using a Cytomation MoFlo instrument. Data were analyzed using FlowJo (Tree Star). To assess the effects of RNA-mediated interference (RNAi) on target mRNA levels, Northern analysis was performed. Total RNA was isolated using the Trizol reagent (Sigma, St. Louis) and processed as described (Sambrook *et al.* 1989). Probes to mRNA were chosen so as to not overlap with the region of the mRNA targeted by the dsRNA in the RNAi experiments.

RESULTS

Overexpression of known negative cell-cycle regulators causes reduced eye phenotypes: To identify genes that function in restricting tissue growth, we used the *ey-GAL4* driver line (Hazelett *et al.* 1998) to overexpress genes represented in the EP collection in the developing eye imaginal disc. The *ey-GAL4* driver line expresses GAL4 under the control of an enhancer element derived from the *eyeless* (*ey*) gene (Hauck *et al.* 1999). As a consequence, GAL4 is expressed ubiquitously in the eye imaginal discs throughout early larval development when all the cells are proliferating. During the third larval instar, expression is restricted to the cycling cells anterior to the morphogenetic furrow.

To examine the effects of expressing known negative regulators of the cell cycle in the developing eye imaginal disc, we first crossed flies carrying UAS-*dacapo* (de Nooij *et al.* 1996), UAS-*Rbf* (Du *et al.* 1996), or UAS-*p21* (de Nooij and Hariharan 1995) to flies expressing *ey-GAL4* and examined the eyes of the progeny. Expression of each of these genes in the proliferating cells of the eye imaginal disc gave rise to adult eyes that are reduced in size when compared to eyes of wild-type flies

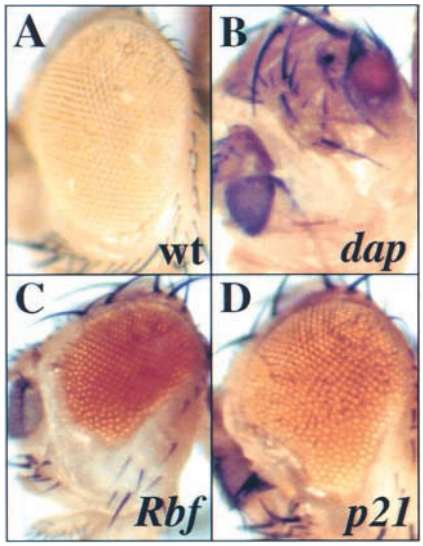

Figure 1.—Adult eye phenotypes resulting from *ey-GAL4*-directed expression of known negative regulators of the cell cycle. (A) Wild-type eye. (B) Overexpression of *dap* results in formation of severely reduced eyes whereas overexpression of either *Rbf* (C) or the human CKI p21 (D) results in a less severe reduction of eye size.

(Figure 1). An examination of eye imaginal discs from third instar larvae showed that these discs were also smaller than wild-type discs (data not shown), indicating that *ey-GAL4*-induced expression of negative cell-cycle regulators inhibited disc growth during eye development. Notably, there was significant phenotypic variation within the progeny of each cross, ranging from slightly reduced eyes in some flies to very small eyes in others. This variation is difficult to explain and may reflect either differences in the levels of GAL4 expression in individual eye imaginal discs or the influence of subtle environmental factors that affect the phenotypes. Nevertheless, these test crosses demonstrate that the overexpression of known negative cell-cycle regulators under *ey-GAL4* control reduced eye size and suggested that novel negative regulators of growth and proliferation may be identified by overexpressing them in the eye disc under *ey-GAL4* control.

Identification of novel negative cell-cycle regulators in Drosophila through a gain-of-function screen: To identify novel regulators of tissue growth, each EP line was crossed to flies expressing *ey-GAL4* and the subsequent progeny were screened for reduced eye size. We tested 2296 individual EP lines in this manner. Fifty-three (2.3%) of the lines displayed phenotypic abnormalities. Of the 53 EP lines, 3 lines displayed *ey-GAL4*-dependent lethality; 4 lines gave slightly rough eyes; and 46 EP lines showed reduced eye size of varying severity (Table 1). The lethality is likely due to low levels of *ey-GAL4*-driven EP expression in other tissues that perturbs the development of essential organs and affects organism viability.

A major advantage of performing a screen utilizing the EP lines is that the *P*-element insertion in most of the lines has been identified with respect to the completed genomic sequence. Thus the genes adjacent to the site of insertion of the *P* element can be identified rapidly. The 46 EP lines identified from the screen as having *ey-GAL4*-dependent reduced eye phenotypes represent insertions at 32 different genetic loci (Table 1). Thirteen lines contain insertions in known genes. As expected, some of these, including *hedgehog* (*hh*) and *decapentaplegic* (*dpp*), have well-characterized functions in eye development. Other genes identified include regulators of the cytoskeleton (*Rac2* and *pebble*) and *fringe* (*fng*), a modulator of extracellular signals. Also identified were *Kruppel-homolog 1* (*Kr-h1*) and *elbowB* (*elB*), two putative transcriptional regulators. The remaining 19 loci have conceptual ORFs that have been identified by the sequencing project. Not all insertions were located in the 5′ region of the ORFs. In one instance, the EP2340 line, the EP element inserted within the coding region of the gene and is likely to generate a protein with an N-terminal truncation when compared to its wild-type counterpart. In two instances, EP1595 and EP2419, the EP elements had inserted downstream of the coding region so as to direct antisense transcription. Thus, for those two lines, the *ey-GAL4*-dependent reduced eye phenotypes may represent a partial or complete loss-of-function phenotype.

From the primary screen, we identified 32 genes that, when overexpressed in the developing eye imaginal disc, result in a reduced eye phenotype. In addition to identifying genes with known functions in eye development, we also identified previously characterized genes that hitherto had not been studied in the context of eye development. Finally, we showed that increased expression of 19 novel ORFs also results in a small eye phenotype.

Further classification of genes identified in the screen: Overexpression of genes can perturb eye development in a variety of ways. We are most interested in identifying genes that restrict cell growth and cell proliferation. Another category of genes likely to be identified in this screen includes those that, when overexpressed, are toxic to cells and result in cell death. To help identify this class of genes and to help define some of the mechanisms by which overexpression results in a reduced eye phenotype, we tested the identified lines in three other ways.

We reasoned that a subset of the genes identified may reduce eye size because overexpression of these genes is toxic and kills cells. If so, then overexpression of these genes is likely to affect both cycling cells and postmitotic cells. In contrast, genes that perturb cell proliferation are more likely to have an effect only in cycling cells. To help distinguish between these two classes of genes, we crossed the selected EP lines to a *sevenless*-GAL4 (*sev-GAL4*) driver line. The *sev-GAL4* driver directs expres-

TABLE 1

List of EP lines that generate a small eye phenotype using the *ey-GAL4* driver

	Map position	Rørth line	Gene	Description	*ey-GAL4*	*sev-GAL4*	*en-GAL4*	Suppression by *cycE*
1	2B16-18	1232	*CG3600*		++	Rough, slightly glassy	Semilethal; wing folds	No
2	3C2	3617	*CG2766*		++	wt	wt	Sup
3	5B1-2	1349	*CG12410*	Twisted gastrulation protein precursor	+	wt	wt	—
4	7A1-5	1617	*CG9650*	C_2H_2 Zn finger	+++	Lethal	Lethal	No
5	8F8-10	1149	*CG15321*	CREB-binding homolog	+++	Lethal	Lethal	No
		1179			++	Glassy, semilethal	Lethal	No
6	12A8-10	1335	*CG11172*		++	Mildly rough	Lethal	Sup
		1390			+++	Mildly rough	Venation defects	Sup
		1508			+++	Mildly rough	wt	No
7	12D1-2	1595	*CG11068*		++	Lethal	Lethal	—
8	21B1-2	456	*CG11371*		+	Slightly rough	Lethal	—
9	22D1-2	598	*aop/yan*	ETS domain transcription factor	Lethal	wt	Incomplete venation	—
		2500			Lethal	wt	Incomplete venation	No
10	26B4	2289	*Kr-h1*	*Kruppel* homolog; C_2H_2 Zn finger	+++	Glassy	Lethal	No
11	22F1-2	2232	*DPP*	TGFβ receptor ligand	+++	Lethal	Lethal	No
12	24E3-4	578	*Traf1*	TNF-receptor associated factor 1	++	wt	wt	Sup
13	28B1-2	2419	*CG13791*		Rough	Rough	wt	—
14	34A5-6	2317	*CG9332*	TPR domain	+++	Lethal	Lethal	Sup
15	35B2-3	965	*elbowB*	C_2H_2 Zn finger	++	wt	Incomplete venation	Sup
		2039			+++	wt	Lethal	Sup
16	35D1	633	*escargot*	C_2H_2 Zn-finger transcription factor	+	Lethal	Lethal	—
		684			+	Lethal	Scalloped wings	No
		2009			+++	Lethal	Scalloped wings	No
		2408			+	Lethal	Scalloped wings	—
17	39B3-4	2490	*Hr39*	Nuclear hormone receptor-like in 39	+++	wt	Blisters, reduced wing	No
18	43A2-3	2340	*INCENP*	Chromosomal passenger protein	+++	wt	Smaller posterior compartment; reduced number of cells	Sup
19	46D1-2	2162	*CG15862*	CNMP-binding site; cAMP-dependent kinase	+	wt	wt	Sup
20	47D5-6	2359	*shnurri*	C_2H_2 Zn-finger transcription factor	+++	Rough	Venation defects	Sup
21	50C20-23	2054	*CG6701*	C_2H_2 Zn finger; pro-rich domain	+++	Mildly rough	Some reduced wings; incomplete penentrance	Sup
22	57A5-6	2586	*CG13432*	KH domain ribonucleoprotein	Rough	Lethal	Lethal	—
23	62A1-2	3704	*Rhomboid*	Intracellular serine protease	Rough	Glassy	Lethal	—
24	62A4	3673	*CG12084*	ARM repeat	++	wt	wt	Sup

(*continued*)

TABLE 1

(Continued)

	Map position	Rørth line	Gene	Description	*ey-GAL4*	*sev-GAL4*	*en-GAL4*	Suppression by *cycE*
25	63C1-2	714	*CG14959*		+	wt	wt	—
		3041			+++	wt	wt	No
		3139			+	wt	wt	—
		3560			++	wt	wt	No
		3689			+++	wt	wt	No
		3718			+	wt	wt	—
26	63D1	3725	*YT521-B*	Pro-rich domain	+	wt	wt	—
27	65F10	3118	*Rac2*	Rho family GTPase	+++	Rough	Venation defects, blisters	Sup
28	66A17-18	3415	*pebble*	Guanyl-nucleotide exchange factor	+++	Rough	Few incomplete veins	Sup
29	66F5-6	3659	*CG4727*		+	Rough	Smaller posterior compartment; Reduced number of cells	—
30	67D10	3083	*CG12362*	Ring finger; IBR	+	wt	wt	No
		3523			+++	wt	wt	No
31	69E1-2	3622	*CG17667*	LRR; transmembrane receptor	Lethal	Rough	Venation defects	Sup
32	78A1-2	3082	*fringe*	UDP-glycosyltransferase	+++	wt	Reduced wings	Sup
33	78C3-4	3468	*Eip78C*	Ligand-dependent nuclear receptor	Rough	wt	wt	—
34	88D1-2	666	*CG7552*	WW domain; ATP/GTP binding site	+	wt	wt	Sup
		747			++	wt	wt	No
35	94E1-2	3521	*hedgehog*	Ligand for *patched*	++	wt	wt	No
36	95E7-F1	601	*CG6268*	Ankyrin-like	+++	wt	wt	No
37	98F13-14	3390	*CG11873*		+	Lethal	wt	—
38	100C6-7	1076	*CG11518*	PHD zn-finger transcription factor	++	wt	Reduced wing	Sup

Lines are ordered by map position. Multiple lines that contain insertions at the same loci are grouped together. Genes of putative coding regions downstream of the EP element were identified using the GADFLY database of the BDGP. Previously characterized genes are underlined. The presence of particular motifs and sequence similarities is based on information found in FlyBase and by BLAST searches. The severity of reduced eye size is indicated by +++ (strongly reduced), ++ (somewhat reduced), and + (slightly reduced). Also indicated are the results of phenotypes generated using *sev-GAL4*, *en-GAL4* and the effect of *cycE* overexpression on the *ey-GAL4*-driven phenotype. Crosses using the *GAL4* drivers were performed at 28° (*ey-GAL4* and *ey-GAL4*, UAS-*cycE*), 25° (*sev-GAL4*), and 26° (*en-GAL4*). For the suppression of the *ey-GAL4*-driven phenotype by *cycE* overexpression, "Sup" indicates suppression, "No" indicates slight or no observable suppression, and "—" indicates that the EP line was not tested. wt, wild type; cNMP, cyclic nucleotide; CREB, cAMP-response element binding; ETS, E26; TPR, tetratricopeptide repeats; ARM, armadillo; IBR domain, in-between RING-fingers domain; LRR, leucine-rich repeat; KH, hnRNP K homology.

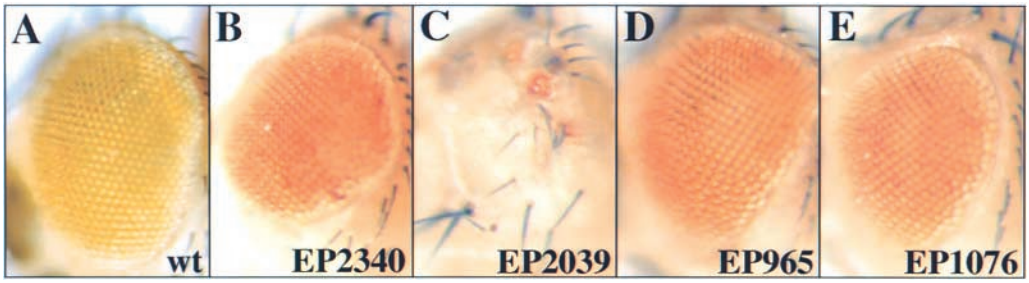

Figure 2.—Adult eye phenotypes resulting from *ey-GAL4*-directed overexpression of EP lines. (A) Wild-type eye. (B) EP(2)2340. (C) EP(2)2039. (D) EP(2) 965. (E) EP(3)1076. Each EP line was crossed to flies carrying the *ey-GAL4* driver at 28°.

sion of GAL4 specifically in the postmitotic cells of the eye imaginal discs. Expression of *p21*, *Rbf*, and *dacapo* UAS transgenes under *sev-GAL4* control resulted in wild-type adult eyes, demonstrating that overexpression of known negative regulators of the cell cycle has no discernible effect on postmitotic cells (data not shown). Of the 32 genes tested with the *sev-GAL4* driver, ectopic expression of 9 genes generated phenotypically abnormal eyes while 16 genes gave wild-type eyes (Table 1). Thus insertions in 9 of 32 loci have an effect in postmitotic cells, indicating that the overexpressed gene may be toxic to all cells or, alternatively, may perturb the growth or differentiation of postmitotic cells. For 16 of 32 loci, overexpression in postmitotic cells shows no effect, suggesting that overexpression of these genes may be able to interfere only with the function of actively cycling cells. Surprisingly, a significant number of loci (7 of 32 loci) exhibited lethality in combination with the *sev-GAL4* driver. This could be due to leaky expression in essential organs.

A second way to subdivide the genes identified in the screen was to determine whether the effect generated by overexpression was tissue specific. It is possible that some of the genes identified in the screen may disrupt processes that are specific to eye development. Thus overexpression of these genes in other tissues may not give a detectable phenotype. In contrast, overexpression of known negative regulators of the cell cycle has been shown to inhibit the proliferation of a variety of cell types. The *engrailed*-GAL4 driver (*en-GAL4*), which directs expression in the posterior compartment of the wing (Neufeld *et al.* 1998), was crossed to each EP line to determine if EP-dependent ectopic expression can result in phenotypic abnormalities in the wing. Expression of *Rbf* and *dacapo* reduced cell division in the posterior wing compartment (data not shown). Of the EP genes tested using the *en-GAL4* driver, 13 showed a wild-type phenotype, whereas 10 showed mutant wing phenotypes and 9 exhibited *en-GAL4*-dependent lethality (Table 1). The phenotypic abnormalities observed included venation defects, wing blistering, and a reduction in wing size. Thus, the majority of genes identified in the screen have effects when overexpressed in tissues other than the eye.

A third method used to classify the lines was aimed at establishing a link with cell-cycle regulation by testing for the ability of *cyclinE* (*cycE*) overexpression to rescue the small eye phenotype. It has previously been shown that the effect of *dacapo* overexpression in the embryo can be overcome by coexpression of a positive regulator, *cycE* (Lane *et al.* 1996). Similarly, in our experiments, coexpression of *cycE* was able to rescue the small eye phenotype induced by *dap* or *Rbf* overexpression (data not shown). We tested the 26 genes that gave the strongest small eye phenotypes for suppression by the coexpression of *cycE*. Of the 26 genes tested, 14 genes showed some rescue of the mutant eye phenotype by *cycE* coexpression whereas 11 genes did not. The extent of suppression differed with each line. Very few lines suppressed the phenotype completely. A major factor was the strength of the *ey-GAL4*-dependent small eye phenotype. Another factor that may influence suppression could be the difference in the level of cyclin E *vs.* EP expression. The suppression by cyclin E was usually strongest when the small eye phenotype was weak. For one gene (*CG7552*) that has multiple EP insertions, the EP line with the strongest eye phenotype was not suppressed by cyclin E coexpression whereas the line associated with the weaker phenotype was suppressed.

In summary, we identified 32 genes that, when overexpressed in the developing eye imaginal disc, result in a reduced eye phenotype. Further classification of these genes showed that 23 of the 32 genes have a *sev-GAL4*-dependent phenotype, suggesting that these genes may cause toxicity in postmitotic cells or have roles in differentiation. In addition, 19 of the genes appear to have functions in wing development, as demonstrated by their *en-GAL4*-dependent phenotypes. Finally, by their ability to show suppression of the reduced eye phenotype by coexpression of cyclin E, 14 genes appear to genetically interact with a regulator of G1-S progression.

Identification of loci encoding potential negative regulators of cell proliferation: We conducted a more detailed analysis of four lines that together represent three different loci. From the secondary analysis of the selected EP lines, four lines (EP965, EP2039, EP2340, and EP1076) that seemed more likely to overexpress negative regulators of growth or proliferation emerged. Expression of these lines under *ey-GAL4* control results in reduced eye size (Figure 2) but gives no detectable phenotype when expressed under *sev-GAL4* control, suggesting that overexpression does not result in toxicity

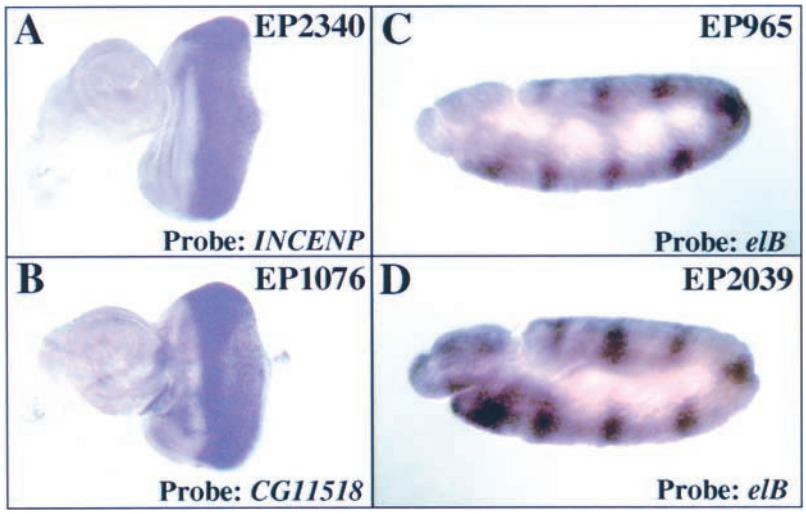

Figure 3.—RNA *in situ* hybridizations performed on third instar eye imaginal discs using the *GMR-GAL4* driver (A and B) or embryos using the *prd-GAL4* driver (C and D) and probed with digoxigenin-labeled riboprobes derived from the open reading frame downstream of the insertion site. Templates for the transcription reactions were performed using cDNAs of the predicted genes in A and B. For C and D, a subcloned fragment of the putative coding region was used as template. For each experiment, the sense RNA riboprobe control did not show any detectable signal (data not shown). The genotypes are (A) +; EP2340/*GMR-GAL4*; +, (B) +; *GMR-GAL4*/+; EP1076/+, (C) +; EP965/+; *prd-GAL4*/+, and (D) +; EP2039/+; *prd-GAL4*/+.

to all cells. Second, *en-GAL4*-directed expression of these lines results in a reduced number of cells in the posterior compartment of the wing, indicating a general effect of these genes on cell growth and cell proliferation. Third, coexpression of *cycE*, a positive regulator of the cell cycle, can suppress the reduced eye size generated by overexpression of these genes alone. We therefore reasoned that these genes may function to regulate cell-cycle progression during Drosophila development. The four lines chosen represent insertions at three loci: INCENP, *elB*, and the gene designated *CG11518*.

To establish that the predicted ORFs downstream of the respective EP insertions were indeed being expressed ectopically in the presence of *GAL4* drivers, digoxigenin-labeled antisense RNA probes were made using the cDNA corresponding to INCENP and *CG11518*. In the case of *elB*, a cDNA clone was not available and therefore a fragment of the predicted third exon was amplified by PCR and used as the template to generate antisense RNA probes. In the presence of *GMR-GAL4* (directing GAL4 expression behind the morphogenetic furrow in the eye imaginal disc), the antisense probes specifically detected expression of the INCENP and *CG11518* in the expected pattern in the EP2340 and EP1076 lines, respectively, confirming that these genes were indeed overexpressed in those EP lines (Figure 3, A and B). Because of the apparent high and ubiquitous level of *elB* expression in the eye imaginal disc (data not shown), we looked at specific GAL4-directed *elB* expression during embryogenesis in the EP965 and EP2039 lines. In both cases, the *elB* antisense probe demonstrated expression of the *elB* RNA in the striped *paired* pattern in embryos (Figure 3, C and D).

Characterization of growth and cell proliferation in the EP2340 line: The *P* element in the EP2340 line is inserted within the coding region of the gene encoding INCENP. Originally identified in a screen that utilized monoclonal antibodies to identify proteins tightly associated with the chicken mitotic chromosome scaffold (Cooke *et al.* 1987), INCENP is a chromosomal passenger protein that appears to play several roles during mitosis. It is concentrated at the centromeres at the start of mitosis, moves to the spindle midzone during anaphase, and later translocates to the cleavage furrow during cytokinesis (Cooke *et al.* 1987). INCENPs from various organisms are identified by a conserved motif at the C-terminal region, the IN box (R. R. Adams *et al.* 2000; Uren *et al.* 2000). In vertebrates, INCENPs are highly conserved through the entire sequence. The N-terminal region contains motifs for targeting the protein to the centromere and association with heterochromatin proteins and tubulin (Ainsztein *et al.* 1998; Mackay *et al.* 1998; Wheatley *et al.* 2001). In Drosophila, the gene *CG12165* encodes the INCENP and has the characteristic "INCENP box" at the C terminus of its amino acid sequence (Figure 4A). The N-terminal portion of Drosophila INCENP does not show obvious sequence similarity to the highly conserved vertebrate INCENPs.

Because the EP element is inserted within the INCENP coding region (Figure 4A), transcripts generated from the *P* element would result in the translation of a protein that lacks the first 119 amino acids. In vertebrates, it has been shown that the N-terminal region is required for proper targeting of INCENP during mitosis. Despite the divergence in sequence, it is likely that the N-terminal domain of Drosophila INCENP has a similar function. Thus it is conceivable that the inhibition of tissue growth in the EP2340 line may have resulted from the overexpression of an INCENP protein with an N-terminal truncation. To test whether overexpression of wild-type INCENP also affected tissue growth, we generated transgenic flies carrying a gene encoding full-length INCENP under the control of *GAL4*-responsive UAS elements. In the presence of *ey-GAL4*, overexpression of full-length INCENP resulted in a small eye phenotype (Figure 4C) indistinguishable from that observed in the EP2340 line, as compared

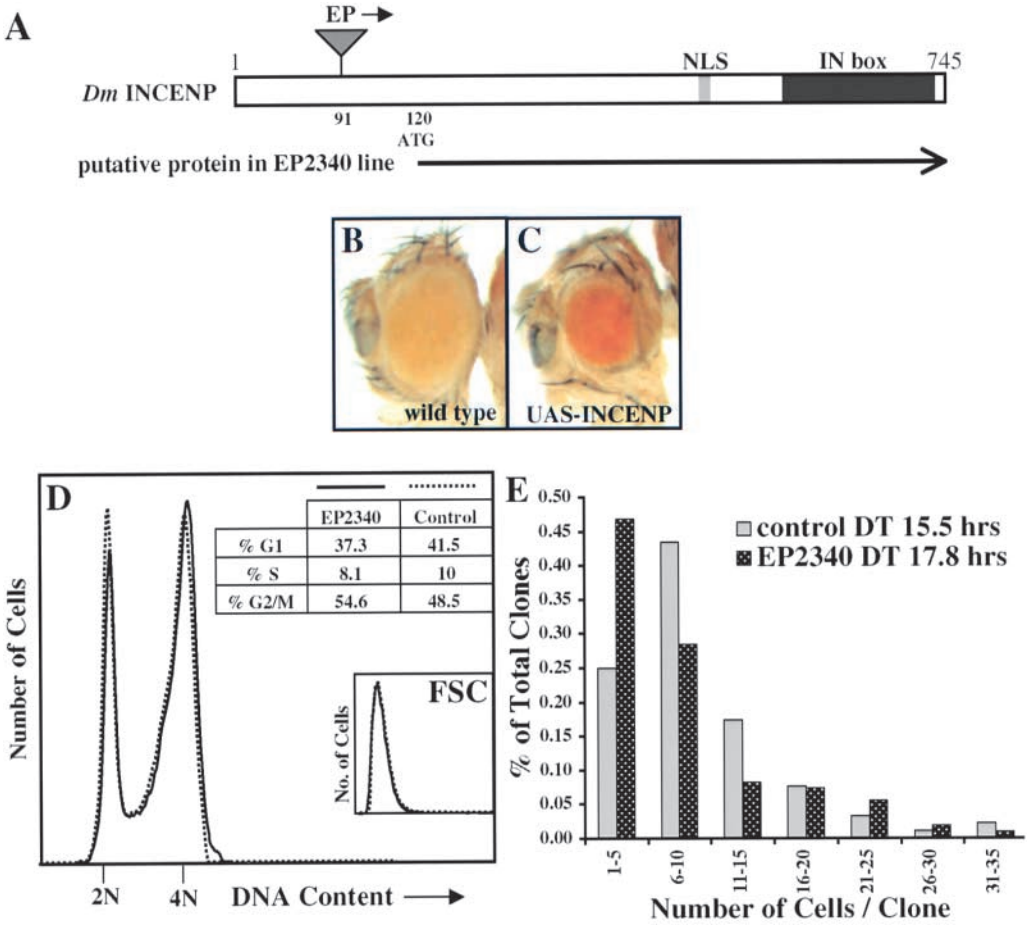

FIGURE 4.—(A) Schematic representation of the protein encoded by the gene downstream of the EP2340 insertion, *CG12165* (INCENP). The site of the EP insertion is at the ninety-first codon. The C-terminal portion contains the INCENP box motif present in all INCENP homologs. Since the EP insertion occurs at codon 91, the expressed protein is expected to begin at the ATG at codon 120. (B and C) Overexpression of full-length wild-type Drosophila INCENP under *ey-GAL4* control also results in a reduced eye size. (D) DNA content and forward scatter (FSC) plots of third instar wing imaginal disc cells expressing p35 (control: dashed line) and p35 + EP2340 (solid line). (E) Histogram indicating the number of cells and clone sizes overexpressing either p35 alone ($n = 92$) or p35 + EP2340 ($n = 109$) as a ratio of the number of clones of that category to the total number of clones.

to control eyes (Figure 4B). Thus, it is likely that the N-terminally truncated INCENP produced by EP2340 overexpression functions in this situation in a manner similar to its full-length counterpart.

To characterize the proliferative properties of cells expressing EP2340, we examined cell size and cell-cycle phasing in green fluorescent protein (GFP)-marked clones of cells in the wing imaginal disc and compared their properties to control cells expressing GFP. Expression of EP2340 did not have any observable effect on cell size as assessed by the forward scatter parameter. However, EP2340 overexpression resulted in a small increase in the proportion of cells with a 4N DNA content (cells presumed to be in G2; Figure 4D). We also determined the population doubling time of cells overexpressing EP2340. A potential pitfall in doubling time measurements is the effect of cell competition, a phenomenon in which growth-disadvantaged cells are eliminated by apoptosis. Hence, to eliminate cell death, all doubling times were measured while coexpressing the caspase inhibitor, *p35* (HAY *et al.* 1994). The population doubling time was 17.8 hr in cells overexpressing EP2340 and p35, which was 15% longer than the doubling time of 15.5 hr calculated for control cells expressing p35 alone (Figure 4E). An unchanged cell size and an increased doubling time suggest that the rate of growth (mass accumulation) is reduced in the mutant cells and that the increased doubling time keeps pace with the reduced growth rate.

To assess the consequences of reducing INCENP function we used RNAi in SL2 cells in culture (CAPLEN *et al.* 2000; CLEMENS *et al.* 2000; HAMMOND *et al.* 2000). Double-stranded RNA of 700 bp derived from the INCENP cDNA was added to the culture medium. The cells were examined 4 days later. By this stage no INCENP RNA was detectable by Northern blot analysis (Figure 5E). Cells treated with INCENP dsRNA had a significant increase in 4N cells at the expense of cells with a 2N DNA content when compared to the control population (Figure 5, G and H). Furthermore, cells with a DNA content corresponding to 8N accounted for 38.5% of the population. The size of the treated cells, as assessed by forward scatter, was larger than wild-type cells (Figure 5F). Thus a reduction in INCENP function leads to an accumulation of large polyploid cells. We also examined embryos that were homozygous for the INCENP loss-of-function mutation. In contrast to wild-type blastoderm embryos after cellularization (Figure 5, A and B), cell outlines in mutant embryos (Figure 5, C and D) are irregular and appear to contain increased amounts of DNA as assessed by staining with the DNA-binding dye, YOYO. These observations correlate with our findings using RNAi in SL2 cells.

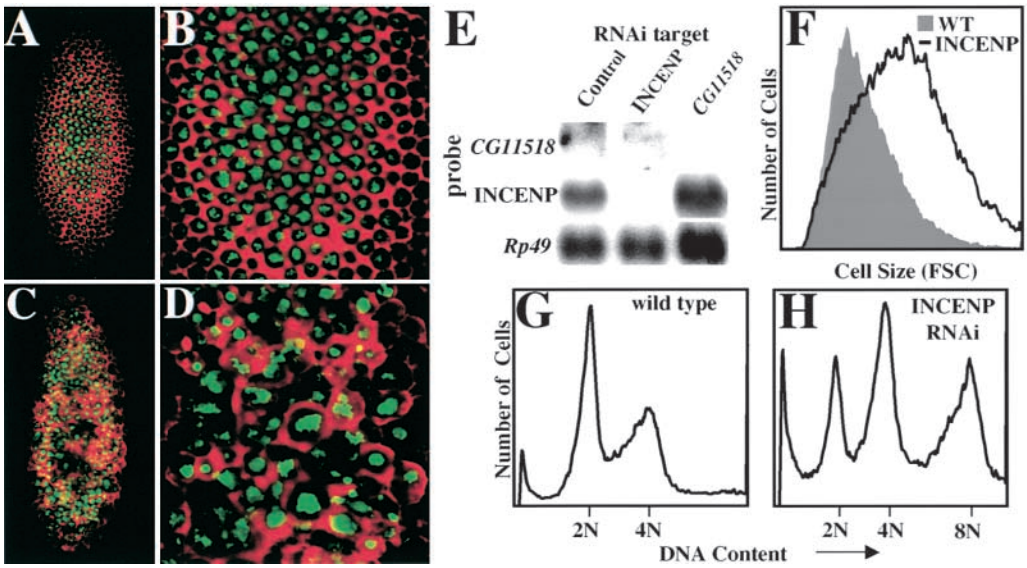

FIGURE 5.—(A–D) Cellularized blastoderm embryos stained with YOYO and anti-actin antibody to visualize DNA and cell outlines. Anterior is at the top. (A and B) Wild-type embryos show a regular array of cells. (C and D) Embryos homozygous for the EP2340 insertion show large and irregular cells with an increase in DNA staining. (E) Northern analysis of SL2 cells treated with dsRNA. (G and H) Comparison of the DNA content of SL2 cells and those where INCENP levels are reduced using RNAi. The FSC profiles are compared in F.

Characterization of insertions at the *elbowB* locus: The lines EP965 and EP2039 have insertions that are 171 bp apart and in the same orientation, ~16.7 kb upstream of the gene, *elB* (*CG4220*). Even though no cDNA has been identified for this gene so far, the EP2039 insertion was recently shown to be a weak *elB* allele, and a putative open reading frame (*BG:DS 06238.3*) was identified by the Berkeley Drosophila Genome Project (ASHBURNER *et al.* 1999). This gene is predicted to encode a zinc-finger protein (Figure 6A) and shows 27% homology to *no-ocelli* (*noc*; CHEAH *et al.* 1994), a gene 100 kb proximal to *BG:DS06238.3* (ASHBURNER *et al.* 1999). The function of *elB* is unknown. No other transcription units have been identified between the EP insertions and *elB*. Both EP965 and EP2039 expression increased levels of *elB* RNA in a *GAL4*-dependent manner (Figure 3, C and D).

We examined the properties of proliferating cells in the wing imaginal disc in the EP2039 line. Compared to the wild-type control, overexpression of the EP2039 line did not have any observable effects on cell size as determined by forward scatter (Figure 6B). However, EP2039 overexpression did result in a slight change in cell-cycle phasing. There is a small but reproducible increase in the G2/M population (Figure 6B), suggesting that EP2039 overexpression may be able to restrict either entry into or passage through mitosis. We determined the population doubling time of cells overexpressing EP2039. Overexpression of EP2039 and p35 resulted in a doubling time of 18.6 hr, which was 26% longer than the doubling time of 14.8 hr calculated for the control cells expressing p35 alone (Figure 6C). An increased doubling time with no change in cell size is consistent with a decreased rate of growth (mass accumulation) with a concomitant slowing of the cell cycle.

RNAi was used to reduce the levels of *elB* in SL2 cells. *elB* is expressed at extremely low levels in SL2 cells. Expression could not be detected by Northern blotting (data not shown), but could be detected at low levels using Affymetrix oligonucleotide microarrays (O. STEVAUX, D. DIMOVA and N. DYSON, personal communication). There was no change in the growth properties of the treated SL2 cells, although it is important to point out that we could not monitor the change in the level of *elB* expression (data not shown). We also considered the possibility that *elB* and *noc* might have shared functions. However, RNAi experiments using dsRNA for both *elB* and *noc* showed no effect on cell division (data not shown).

Characterization of the EP1076 insertion: The line EP1076 contains an EP insertion in the 5′-UTR of the transcription unit designated *CG11518*. It encodes an 815-amino-acid protein containing a plant homology domain (PHD) zinc-finger motif (Figure 7A) in its C-terminal region (amino acids 750–802). This motif is found in the polycomb and trithorax group genes in Drosophila (AASLAND *et al.* 1995). These genes encode proteins that are involved in regulating the expression of homeotic genes by changing chromatin structure (PARO and HOGNESS 1991; TAMKUN *et al.* 1992). Thus the PHD finger, a C_4HC_3 zinc-finger motif, is thought to function in chromatin-mediated transcriptional regulation. A BLAST search identified proteins in other organisms, including mammals, that display a high degree of sequence similarity in the PHD zinc-finger domain (Figure 7A).

In cells of the wing imaginal disc, overexpression of EP1076 did not have any observable effects on cell size as assessed by forward scatter (Figure 7B). However, EP1076 overexpression also resulted in a small increase in the population of cells with a 4N DNA content (Figure 7B). Cells overexpressing EP1076 and p35 have a doubling time of 17.5 hr, which is 10% longer than the doubling time of 15.9 hr calculated for control cells expressing p35 alone (Figure 7C). Thus, overexpression appears to result in a small decrease in the growth rate

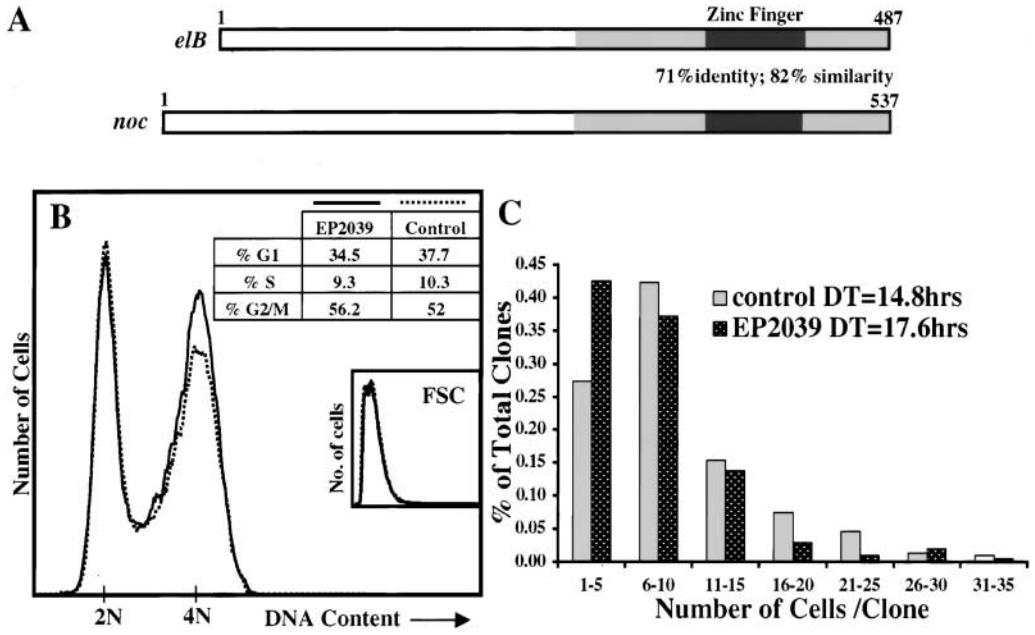

FIGURE 6.—(A) Schematic representation of proteins encoded by the putative gene, *elB* and its paralog, *noc*. Within the zinc-finger region, the amino acid sequence shows 71% identity and 82% similarity (solid bars). A lower degree of sequence similarity is observed over the entire C-terminal region (shaded bars). (B) DNA content and forward scatter (FSC) plots of cells dissociated from third instar wing imaginal disc cells expressing p35 (control: dashed line) and p35 + EP2039 (solid line). (C) Histogram showing clone sizes of cells overexpressing p35 alone (n = 215) or p35 + EP2039 (n = 202).

of the population. Since overexpression of cyclin E was able to restore normal eye size in this EP line, we examined the properties of the "rescued" cells. Overexpression of cyclin E does not restore normal cell-cycle phasing. Cells overexpressing both cyclin E and EP1076 behave much like cells overexpressing cyclin E alone. More than 80% of cells have a 4N DNA content. However, coexpression of cyclin E reduces doubling time by 12% when compared to cells expressing EP1076 alone and the cell size is slightly reduced (data not shown). Thus cyclin E appears to restore eye size by restoring growth and not by correcting the cell-cycle abnormalities induced by EP1076 overexpression.

Using RNAi, we reduced the levels of *CG11518* RNA in SL2 cells (Figure 5E) and examined their proliferative properties. There was no discernible effect on cell division or growth (data not shown).

DISCUSSION

To identify genes that restrict tissue growth *in vivo*, we screened for genes that, when overexpressed, reduce the size of the Drosophila eye. A screen based on overexpression may identify genes that are missed in screens that depend on phenotypes elicited by loss-of-function mutations, especially in cases in which those phenotypes are subtle or in which the gene has a redundant function. After screening 2296 EP lines using the *ey-GAL4* driver to direct gene expression to proliferating cells of the eye imaginal disc, we identified 53 lines (2.3%) that resulted in a reduction in eye size. Screens using other *GAL4* driver lines have elicited phenotypes in 2–7% of the lines screened (RØRTH *et al.* 1998; ABDELILAH-SEYFRIED *et al.* 2000; HUANG and RUBIN 2000; KRAUT *et al.* 2001). Thus, the number of lines identified in our screen is comparable to the numbers identified in these other screens. The collection of EP lines currently available is thought to account for <10% of the genes in the entire genome (RØRTH *et al.* 1998). In our screen, 46 of the 2296 EP lines generated a reduced eye phenotype. If one were to assume that ~50% of the EP lines were oriented with the promoter toward the immediately adjacent ORF, this would imply that 4% of appropriately oriented insertions (46 of 1148) would generate a phenotype. This is a rough estimate of the percentage of genes that can generate this phenotype since there are many instances where more than one EP line has an insertion in the same locus. The genome sequencing effort identified 13,600 ORFs in the entire genome. On the basis of the number of lines identified in our screen, we would predict that a screen of the entire genome would identify on the order of 500 genes that could generate this phenotype.

Screens that have been conducted by others in some instances have sought phenotypes that are elicited by disrupting cellular processes that are very different from those that regulate disc growth. In one screen, a panneural *GAL4* driver was used to identify genes that disrupted synapse formation (KRAUT *et al.* 2001). Of the 114 lines identified in that screen, only 5 lines were also identified in our screen, indicating that the sets of genes identified in the two screens are largely nonoverlapping. In another screen, overexpression of 105 of 2293 EP lines tested was shown to disrupt the formation of the external sensory (es) organ in the adult (ABDELILAH-SEYFRIED *et al.* 2000). Of the 105 genes, 19 were also identified in our screen. A higher degree of overlap between these two screens is likely to reflect the importance of cell proliferation in generating the es organ and in disc growth as well as a role in patterning genes

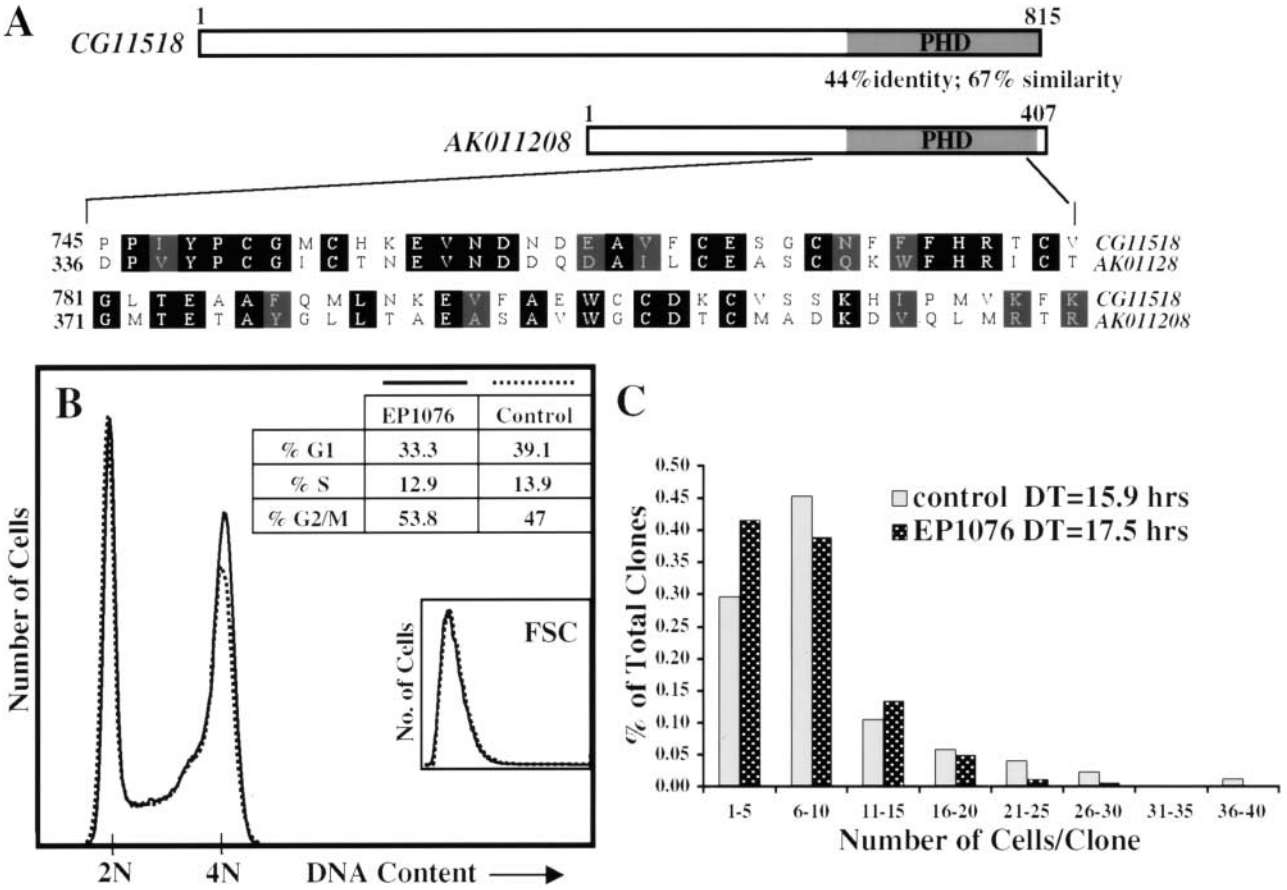

FIGURE 7.—(A) Schematic representation of the protein encoded by the *CG11518* transcription unit. The location of the PHD zinc-finger (C_4HC_3) motif at the C terminus is shaded. A BLAST search revealed that the *CG11518* PHD zinc finger shows sequence similarity to *AK011208*, a putative mouse gene (accession no. BAB27468). (B) Plot showing DNA content and FSC analysis of cells derived from third instar wing imaginal disc cells expressing p35 (control: dashed line) and p35 + EP1076 (solid line). (C) Histogram showing clone sizes of cells overexpressing p35 alone (n = 172) or p35 + EP1076 (n = 188).

(*e.g.*, *hedgehog*) that function in both processes. Thus, the lines identified in our screen, and not in the other screens, may be of particular relevance to the regulation of growth of the eye imaginal disc.

Categories of genes identified: Of the 32 loci identified in the screen, 13 represent known genes. Several of these genes have functions related to cell division. INCENP homologs are important for chromosome alignment and segregation (COOKE *et al.* 1987). Pebble is a guanine nucleotide exchange factor that activates Rho-family GTPases. Mutations in *pebble* result in the accumulation of multinucleate cells due to impaired cytokinesis (LEHNER 1992; PROKOPENKO *et al.* 2000). *escargot* encodes a protein with zinc (Zn) fingers and has been implicated as a negative regulator of endoreduplication in imaginal tissues (HAYASHI 1996a,b). Its overexpression reduces the size of the wing using drivers that express in portions of the wing such as *omb*-GAL4 and *dpp-GAL4* (RØRTH *et al.* 1998). The size of the eye was also reduced dramatically when *sev-GAL4* (a driver that is thought to function only in postmitotic cells) was used. Thus overexpression of *escargot* may be toxic to both cycling and nondividing cells through a mechanism unrelated to its role in regulating endoreduplication. The levels and activities of many genes that function in regulating various aspects of the cell cycle oscillate during the cycle. It is possible that a sustained level of expression of some these genes may be detrimental to cell-cycle progression, cytokinesis, or cell viability.

Among the cell-cycle regulators, it was surprising that line EP2584 was not identified in our screen. This line has an insertion upstream of the *dacapo* gene and was detected in the screen for genes that affected the development of the es organ. We have shown that overexpression of *dap* using the *ey-GAL4* driver and the *UAS-dap* transgene was able to reduce eye size dramatically (Figure 1). Thus it seems likely that the levels of *dap* expressed in the EP(2)2584 line are lower and that this lower level of expression is sufficient to disrupt the formation of the es organ (ABDELILAH-SEYFRIED *et al.* 2000) but not to reduce growth and cell proliferation in the cells of the eye imaginal disc.

Another group of genes identified includes genes

such as *fng*, *dpp*, and *hh*, which function in patterning proliferation and cell fate determination in the eye imaginal disc. For some of these genes, previous studies suggest a likely mechanism of action. For example, *fng* has an important role in patterning cell proliferation in the eye imaginal disc. Expression of *fng* in the ventral half of the eye imaginal disc creates a border of fng^+ and fng^- cells at the equatorial border. This leads to activation of Notch along the border and via an unknown mechanism promotes cell proliferation in the entire disc (Papayannopoulos *et al.* 1998). Eye discs where *fng* expression is either absent or ubiquitous abolish the establishment of the *fng* expression border at the equator, resulting in a failure of Notch activation and a reduction in disc growth (Cho and Choi 1998; Dominguez and de Celis 1998; Papayannopoulos *et al.* 1998).

Our identification of *dpp* in this screen is consistent with the previous observation that overexpression of *dpp* in the eye imaginal disc reduces the number of cells in S phase (Horsfield *et al.* 1998). In our screen, we also identified *schnurri* (*shn*), a cofactor involved in *dpp*-mediated transcriptional activation. It has been shown that in a *trans*-heterozygous combination with a hypomorphic allele of *dpp*, a *shn* mutant allele can suppress the rough eye phenotype caused by a hypomorphic allele of *cycE* (Horsfield *et al.* 1998). The small eye phenotype associated with *hh* overexpression was somewhat surprising, given that it appears to be required for the growth of the eye imaginal disc (reviewed by Treisman and Heberlein 1998). In the third larval instar, *hh* can activate the expression of *dpp* and facilitate progression of the morphogenetic furrow. It can also activate *wingless* expression and block furrow movement. Thus, a perturbation of the normal pattern of *hh* expression is likely to disrupt the orderly movement of the furrow.

Other known genes identified in the screen include signaling molecules (*Kr-H1*, *Rac2*, and *Traf1*) and a receptor (*Hr39*). The *Kr-H1* gene was identified by its sequence homology to *Kruppel*, a Drosophila segmentation gene (Schuh *et al.* 1986). *Kr-H1* contains eight Zn finger domains and is thought to be a transcription factor involved in metamorphosis (Pecasse *et al.* 2000). Overexpression of the associated EP line (*EP2289*) gives strong phenotypes in both the cycling and postmitotic cells of the eye (*ey-GAL4* and *sev-GAL4*) and the wing (*en-GAL4*). Moreover, overexpression of *EP2289* in sensory organ precursor cells resulted in severe loss of sensory cells (Abdelilah-Seyfried *et al.* 2000). Current data indicate that *Kr-H1* functions in cell proliferation and its expression in multiple cell types appears to compromise cell viability. Thus *Kr-H1* may have an uncharacterized role in transcriptional regulation of genes involved in growth or cell survival.

Rac2 is a member of the Rho family of GTPases (Harden *et al.* 1995; Hariharan *et al.* 1995). Overexpression of *Rac2* posterior to the morphogenetic furrow disrupts the eye structure due to the loss of pigment cells and photoreceptors (Nolan *et al.* 1998). Although its paralog, *Rac1*, has been shown to have a positive effect on proliferation in the wing disc (Sotillos and Campuzano 2000), the role of *Rac2* in proliferation has not been explored.

Traf1 is the Drosophila ortholog of the tumor necrosis factor-receptor-associated factor and appears to be an activator of Jun kinase (Liu *et al.* 1999). In the eye imaginal disc, *Traf1* RNA is expressed behind the morphogenetic furrow (Preiss *et al.* 2001), suggesting a role for *Traf1* in maintaining cell-cycle arrest or in differentiation. The overexpression of *EP578* (the EP insertion upstream of *Traf1*) in postmitotic cells of the eye disc did not adversely affect eye development (Table 1). Thus, it is possible that when overexpressed in proliferating eye precursor cells, *Traf1* may drive cells to exit the cell cycle prematurely, resulting in a smaller pool of precursor cells to generate a complete eye. In support of this model is our observation that coexpression of *cycE* can suppress the reduced eye phenotype caused by *EP578* overexpression (Table 1).

Hormone receptor-like in 39 (*Hr39*) encodes a nuclear hormone receptor that acts to repress *alcohol dehydrogenase* transcription (Ayer *et al.* 1993). A *P*-element insertion allele of *Hr39*, when heterozygous, decreases minichromosome inheritance (Dobie *et al.* 2001). So far no role has been described for *Hr39* that pertains to cell proliferation. However, since expression of EP2490 (the EP insertion upstream of *Hr39*) using *sev-GAL4* resulted in wild-type eyes, the small eye phenotype produced when *ey-GAL4* was used is unlikely to result from mere toxicity of the overexpressed protein to cells. Thus, the screens may have uncovered a new role for *Hr39* in regulating cell proliferation.

Properties of INCENP, *elB*, and *CG11518*: We conducted a more detailed characterization of three of the genes that were identified in the screen. The properties of INCENP have been examined in some detail in vertebrate cells (reviewed by Adams *et al.* 2001a) and a recent publication describes some of the properties of Drosophila INCENP (Adams *et al.* 2001b). INCENP is found in a complex with the Aurora-B kinase and is required for its histone H3 kinase activity. Cells lacking INCENP function display defects in the alignment of metaphase chromosomes and incomplete disjunction between sister kinetochores. These cells can assemble a contractile ring but fail to complete normal cytokinesis (R. R. Adams *et al.* 2000; Kaitna *et al.* 2000). INCENP is initially found on condensing chromatin, then in centromeric regions, and then in the central portion of the spindle and the mid-body. It has been proposed that INCENP's major role is to act as a targeting factor for Aurora-B.

Our studies using RNAi in SL2 cells are consistent with the functions described for INCENP; we observe large polyploid cells. However, we have also shown that

overexpression of either full-length or an N-terminally truncated version of INCENP in the eye imaginal disc reduces disc growth and the size of the adult eye. Overexpression of INCENP in cells of the wing disc appears to result in a slowing of cell proliferation as assessed by an increase in the population doubling time. If this were the result of a slowing down of progression through mitosis, one would expect that the rate of growth (mass accumulation) would continue as before and would result in an increase in cell size. However, INCENP overexpression does not appear to change cell size. This indicates that the rate of growth has slowed and there is an equivalent slowing of the cell cycle such that normal cell size is maintained. It is difficult to explain this observation on the basis of the roles described for INCENP in cell division. INCENP binds to microtubules and the overexpression of INCENP in vertebrate cells in culture has been shown to disrupt the microtubule network. This might lead to an impairment of growth in interphase cells as we have observed in cells from the wing disc.

The *elB* gene encodes protein with a Zn-finger motif. It is most similar to the Drosophila gene *noc* (Cheah *et al.* 1994). The line EP2039, which overexpresses *elB*, generates a dramatic small eye phenotype using the *ey-GAL4* driver. The EP965 line generates a weaker phenotype, possibly the result of a lower level of expression. Cells overexpressing the EP2039 insertion have no change in cell size and a considerable increase in the population doubling time (17.6 *vs.* 14.8 hr). This indicates that the rate of growth (mass accumulation) has slowed down with an equivalent slowing of the cell cycle. There is also a change in the phasing with a small increase in the population with a 4N DNA content and a small decrease in the percentage of cells presumed to be in G1 and S phases. Thus the extension of the cell cycle appears to lengthen G2 more than the other phases of the cell cycle, suggesting a possible function for this protein at that stage. When RNAi was used to reduce *elB* function in SL2 cells, there was no perceptible difference in division times (data not shown). A more detailed analysis of *elB* function *in vivo* awaits the generation of complete loss-of-function mutations in the gene.

The *CG11518* gene encodes a protein with a PHD zinc finger that may function in chromatin remodeling. Overexpression of *CG11518* elicits the same phenotype as *elB*, albeit a weaker one. The extension of division time is less pronounced but the change in cell-cycle phasing is of a similar magnitude. As with *elB*, a reduction in its function in SL2 cells using RNAi resulted in no measurable change in doubling time (data not shown). One possible explanation of the phenotypes elicited by overexpression is that both *elB* and *CG11518* may function in repressing the expression of genes that promote growth (mass accumulation).

Given that at least two of these genes appear to reduce growth when overexpressed, it is somewhat surprising that normal growth is restored by cyclin E overexpression. In clones of cyclin E overexpressing cells in the wing disc, cell-cycle phasing is altered but growth does not change (Neufeld *et al.* 1998). However, in accordance with our findings, cyclin E overexpression has also been shown by others to overcome the reduced growth elicited by ectopic overexpression of a variety of transcriptional regulators in the developing eye and head (Jiao *et al.* 2001). We have also previously shown that cyclin E overexpression can antagonize the growth reduction caused by the combined overexpression of Tsc1 and Tsc2 (Tapon *et al.* 2001). Thus although cyclin E, unlike cyclin D, is unable to increase growth in wild-type cells (Neufeld *et al.* 1998; Datar *et al.* 2000), overexpression of cyclin E appears capable of restoring growth in several situations where growth is already compromised. Overexpression of cyclin D can also suppress the small eye phenotype in the EP2039, EP1076, and EP2340 lines (data not shown), presumably by restoring normal growth levels. Interestingly, the small eye phenotypes elicited by overexpression of *dap* or *Rbf* (Figure 1), two genes that are considered to regulate cell-cycle progression and not growth, are suppressed by cyclin D overexpression (data not shown). Thus, under certain conditions at least, cyclin D and cyclin E each appear capable of performing some of the functions normally attributed to the other. In these situations, each of these cyclins could possibly increase the expression, or activity, of the other. The interplay of cyclin D and cyclin E in growth regulation thus appears to be complex and clearly merits further study.

Concluding remarks: We have identified insertions in 32 loci that, when overexpressed, reduce the growth of the eye imaginal disc with a resulting decrease in the size of the adult eye. Flow cytometry and division time estimates of cells that overexpress the EP insertions allowed us to examine the consequences of overexpressing these genes at the cellular level. RNAi now provides a way of assessing the effects of a reduction in gene function on growth and cell-cycle progression in tissue culture cells. Thus the combination of a gain-of-function screen and an examination of the isolated genes using phenotypes induced by RNAi in cell culture is likely to facilitate the identification of more novel regulators of growth and cell proliferation.

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Note added in proof: After the submission of this manuscript, three recent publications have analyzed loss-of-function mutations in *CG11518*. The locus is now named *pygopus* (T. Kramps, O. Peter, E. Brunner, D. Nellen, B. Froesch *et al.*, 2002, Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. Cell **109:** 47–60; D. S. Parker, J. Jemison and K. M. Cadigan, 2002, Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. Development **129:** 2565–2576; B. Thompson, F. Townsley, R. Rosen-Arbesfeld, H. Musisi and M. Bienz, 2002, A new nuclear component of the Wnt signalling pathway. Nat. Cell Biol. **4:** 367–373).

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