A Genetic Screen to Identify Components of the *sina* Signaling Pathway in Drosophila Eye Development

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ABSTRACT

Specification of the R7 photoreceptor cell in the developing Drosophila eye requires the *seven in absentia* (*sina*) gene. We demonstrate that ectopic expression of *sina* in all cells behind the morphogenetic furrow disrupts normal eye development during pupation, resulting in a severely disorganized adult eye. Earlier events of cell fate specification appear unaffected. A genetic screen for dominant enhancers and suppressors of this phenotype identified mutations in a number of genes required for normal eye development, including *UbcD1*, which encodes a ubiquitin conjugating enzyme; *SR3-4a*, a gene previously implicated in signaling downstream of *Ras1*; and a Drosophila homolog of the Sin3A transcriptional repressor.

DURING development of multicellular organisms, cell fate determination is often influenced by interactions between neighboring cells. Our understanding of the molecular mechanisms underlying such interactions has advanced greatly in recent years, and has been aided by a number of model systems. Development of the R7 photoreceptor in the Drosophila compound eye has been a particularly profitable system for dissecting the intercellular signaling pathways used to specify cell fate (reviewed by Wasserman *et al.* 1995; Simon 1994; Zipursky and Rubin 1994). A large number of genetic, molecular, and biochemical studies have led to identification of many of the signaling components required for R7 development, their biochemical functions, and their hierarchy within the R7 signaling pathway.

A primary event of R7 specification is activation at the R7 cell surface of the SEVENLESS (SEV) receptor tyrosine kinase (Tomlinson and Ready 1986). The R7 cell is singled out from a group of five equipotential cells (R7 and four lens-secreting cone cells) by virtue of its contacts with the neighboring R8 photoreceptor, which expresses the ligand for SEV, BRIDE OF SEVENLESS (BOSS; Reinke and Zipursky 1988). Following receptor activation, the signal is passed from the cell surface to the nucleus by a signal transduction pathway common to all eight photoreceptors (Simon *et al.* 1991). Ligand binding leads to activation of the RAS1 protein, which initiates a series of successive phosphorylation events via the mitogen-activated protein kinase

(MAPK) pathway. Activation of this pathway causes activated MAPK to accumulate in the nucleus, where it phosphorylates a number of nuclear proteins that presumably control expression of genes that bring about photoreceptor differentiation. Among such targets of MAPK are the transcription factors JUN and POINTED, whose activities are required for photoreceptor development, and YAN, a negative regulator of photoreceptor fate (Bohmann *et al.* 1994; O'Neill *et al.* 1994; Rebay and Rubin 1995).

An additional nuclear protein required for successful transmission of the RAS-MAPK inductive signal is SINA, a ring-finger protein of unknown biochemical function (Carthew and Rubin 1990). Loss of function mutations in *seven in absentia* (*sina*) prevent normal specification of the R7 cell, and block transformation of cone cells into photoreceptors caused by experimental manipulation of the RAS-MAPK pathway in these cells (Carthew and Rubin 1990; Fortini *et al.* 1992). However, the requirement for *sina* in this pathway is restricted to the R7/cone cell group, as photoreceptors of the R1-6 class are specified correctly in *sina* mutants. Presumably, neuronal induction by the RAS-MAPK pathway in these cells does not require the biochemical activity provided by SINA, or this activity is provided by an alternative component. Although the precise nature of SINA's biochemical activity is unknown, we and others have recently shown that SINA is required for the degradation of TRAMTRACK (TTK), a transcriptional repressor of neuronal cell fate (Tang *et al.* 1997; Li *et al.* 1997). A potential role for SINA in protein degradation also appears to be conserved in vertebrates (Hu *et al.* 1997).

One approach toward further elucidating SINA's function is to identify additional signaling components with which it interacts. For example, two-hybrid and im-

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munoprecipitation studies have revealed that SINA can form a complex with TTK and PHYLLOPOD, a transcriptional target of the RAS-MAPK pathway (Kauffmann *et al.* 1996; Tang *et al.* 1997; Li *et al.* 1997). Genetic interaction screens can provide a powerful complement to these biochemical studies. We have previously identified a number of mutants that interact with a partial loss of function *sina* allele (Carthew *et al.* 1994). In this report we describe a complementary screen for extragenic mutations that modify a phenotype caused by overexpression of *sina* in the developing eye.

MATERIALS AND METHODS

sina misexpression: The *sina* coding region was amplified by PCR and cloned into the *Eco*RI site of pGMR (Hay *et al.* 1994) to create the construct P[*w, GMR-sina*]. Transgenic *GMR-sina* lines were generated by germline transformation. To determine whether functional SINA protein was produced by the P[*w, GMR-sina*] transgene, weak *GMR-sina* lines were tested for the ability to rescue a strong loss of function *sina* mutation. Sections of eyes of the genotype P[*w, GMR-sina*]/+; *sina*[3] revealed that approximately 50% of the ommatidia contained an R7 cell, whereas no R7 cells were observed in *sina*[3] eyes lacking the P[*w, GMR-sina*] transgene.

GMR-sina modifier screen: Isolation, balancing, and chromosomal linkage analysis of dominant enhancers and suppressors of the *GMR-sina* eye phenotype were facilitated by the construction of balancer chromosomes bearing the P[*w, GMR-sina*] transgene. An *X*-linked P[*w, GMR-sina*] insert was mobilized in males carrying the balancers CyO and TM3, and male progeny demonstrating a *GMR-sina* eye phenotype were scored for the subsequent cosegregation of eye phenotype and dominant balancer markers. Two lines were isolated that carry balancer chromosomes with P[*w, GMR-sina*] insertions that provide sufficient levels of *sina* expression to produce a rough eye phenotype suitable for screening. A CyO, P[*w, GMR-sina*] line was used in the modifier screen.

Male *w[1118]* flies isogenic for the second and third chromosomes were fed 25 mM EMS as described (Lewis and Bacher 1968) or treated with 4000 R of X rays (1000 sec at 115 kEV, 5 mA), and then mated to *w[1118]; Adv*/CyO, P[*w, GMR-sina*] females. F1 progeny carrying the CyO, P[*w, GMR-sina*] chromosome and a set of mutagenized chromosomes were assayed for an increase or decrease in the degree of severity of the eye phenotype. Approximately 40,000 (EMS) and 56,000 (X rays) F1 progeny were scored. Putative enhancers and suppressors were backcrossed to the *w[1118]; Adv*/CyO, P[*w, GMR-sina*] stock to test for chromosomal linkage, and balanced over CyO, P[*w, GMR-sina*] or TM3, P[*w, GMR-sina*].

Modifier mutants were mapped meiotically by mating *w[1118]; Adv*/CyO, P[*w, GMR-sina*] males with females carrying a representative allele of each complementation group in *trans* to a multiply marked chromosome. F1 recombinant males with the modifier phenotype were then individually mated to marker females, and the F2 generation was scored for the presence of each marker phenotype. After establishing a meiotic map position for each group, the map positions were further refined by lethal complementation tests using chromosomal deficiencies and P elements from each region, and by cytological examination of polytene chromosomes from X-ray alleles. Non-complementing deficiencies and P elements were as follows: *SS2-1*: P[*w*][1(2)16124] and P[*ry*][1(2)632]; *SS3-1* and *ES3-1*: Df(3R)Tp110 and Df(3R)Antp17; *SS3-3*: P[*w*][1(3)1462], P[*w*][1(3)1782], P[*w*][1(3)6535], P[*w*][1(3)2C8]; *SS3-4*: *SR3-4a[s-192]* and *SR3-*

4a[xs706] (Karim *et al.* 1996); *SS3-5*: Df(3L)81k19 and Df(3l)st-f13; *SS3-7*: Df(3L)AC1 and P[*w*][1(3)2240]; *ES2-1*: Df(2R)Jp1; *ES2-2*: Df(2L)TW84, Df(2L)TW161, P[*w*][1(2)7130], P[*w*][1(2)10523]; *ES2-3*: Df(2R)vg135, Df(2R)vg-C, P[*w*][1(2)7401], P[*w*][1(2)8269]. The map position of *ES1-1* was assigned based on rescue of its lethality by the duplication Dp(1;2)v[656].

sina[4] interaction test: A representative allele of each group was crossed into a *sina[4]* genetic background to generate flies heterozygous for the modifier mutation, and homozygous for *sina[4]*. Ten eyes were sectioned for each group, and scored for percentage ommatidia lacking R7 cells. Tester alleles and percentage R7− ommatidia were as follows: *SS2-1[s35]*, 22%; *SS3-3[s81]*, 17%; *SS3-4[s95]*, 23%; *SS3-6[s297]*, 39%; *SS3-7[s308]*, 39%; *ES1-1[e341]*, 29%; *ES2-1[e1]*, 15%; *ES2-2[e10]*, 19%; *ES2-3[e64]*, 62%; *ES2-4[e220]*, 67%; *ES3-1[e139]*, 11.5%; *sina[4]* homozygotes without a modifier mutation lack R7 cells in 20% of ommatidia. No recombinants were obtained between *sina[4]* and the *SS3-5* locus.

FLP/FRT clonal analysis: One or more representative alleles of each group was recombined onto a centromere-proximal FRT chromosome, and mitotic clones were generated as described (Xu and Rubin 1993). No recombinants were obtained between the *ES3-1* and *SS3-5* groups and their centromere-proximal FRT sites, so we were unable to examine the clonal phenotypes of these groups. In addition, *Star* and *glass* homozygous phenotypes have been described previously (Heberlein and Rubin 1991; Moses *et al.* 1989) and were not analyzed in this study.

Molecular analysis: Plasmid rescue (Pirotta 1986) was used to isolate genomic DNA adjacent to the P element insertions P[*w*][1(2)7401] and P[*w*][1(2)8269] (*ES2-3*); and P[*w*][1(3)1462] and P[*w*][1(3)2C8] (*SS3-3*). These genomic fragments were then used as probes to screen cDNA libraries prepared from eye-antennal imaginal disc (A. Cowman, unpublished results) and embryonic (L. Hong and G. M. Rubin, unpublished results) transcripts. cDNAs that hybridized to genomic DNA on both sides of the P insertion sites were further characterized by sequence analysis. P[*w*][1(3)2C8] was found to be inserted in the 5′ untranslated region of the *UbcD1* gene, 333 nucleotides upstream of the translation start site, between the t1 and t2 *trenino* insertions (Cenci *et al.* 1997). Several related cDNAs that span the *ES2-3* insertions P[*w*][1(2)7401] and P[*w*][1(2)8269] were identified and characterized; the P insertion sites were localized to a common 2 kilobase *Eco*RI fragment at the 5′ end of these transcripts which hybridized to genomic sequences on both sides of the P insertions. Sequence analysis of approximately 1 kilobase of genomic DNA surrounding the insertion sites revealed no sequence in common with that of the cDNAs, indicating the P[*w*][1(2)7401] and P[*w*][1(2)8269] elements are inserted within an intron of this transcription unit. BLAST and PILEUP algorithms (Genetics Computer Group, University of Wisconsin, Madison) were used for protein database searches and amino acid sequence alignments.

RESULTS

sina misexpression phenotypes: SINA protein is normally expressed throughout the developing eye disc, with increased levels in cells that have joined an ommatidial group (Carthew and Rubin 1990). To ask whether this wild-type pattern of *sina* expression is required for normal eye development, we generated transgenic lines bearing a wild-type *sina* gene in the expression vector pGMR, which contains a multimerized segment of the Rh1 promoter that acts as a binding site for the Zn-finger protein GLASS (Hay *et al.* 1994). This

vector provides high levels of *glass*-dependent expression in all eye disc cells posterior to the morphogenetic furrow during larval development, and in all cells except cone cells in the pupal eye (Hay *et al.* 1994).

Thirteen independent *GMR-sina* transformant lines were isolated, each of which displayed eyes with abnormal exteriors, ranging from a slight roughening of the ommatidial lattice to a gross disruption of normal eye morphology (Figure 1, C and E). This range in phenotype is most likely due to variances in expression among the lines, since homozygosing each line resulted in a stronger phenotype. Eyes from strong *GMR-sina* lines were notably smaller and less pigmented than wild-type, and a fusion of ommatidial surfaces resulted in a glazed cuticle covering the eye (Figure 1E). Microscopic examination of sections through such eyes revealed corresponding abnormalities in the underlying retinal cells (Figure 1F). In these lines retinal patterning appeared severely disrupted, and no normal ommatidia were identified. However, no cell types appeared to be lacking, as judged by the presence of pigment granules (pigment cells), lens structures (cone cells), rhabdomeres (photoreceptor cells) and bristles. Sections through eyes from the weaker *GMR-sina* lines with mild exterior phenotypes displayed more subtle de-

fects, including defective ommatidial rotation, lattice disorganization, and occasional missing photoreceptors (Figure 1D). In no case were extra R7 photoreceptor cells observed, indicating that misexpression of *sina* in uncommitted cells in the eye disc is insufficient to direct them into a neuronal program of development.

To determine the developmental stage at which *sina* misexpression results in retinal disruption, we examined the program of ommatidial assembly in larval and pupal eye discs of strong *GMR-sina* lines. In wild-type eye discs the neuronal antigen ELAV is expressed in a characteristic sequence as photoreceptor cells assume their neuronal identities, and expression continues during pupal development in a pattern that anticipates the highly ordered appearance of the adult eye (Robinow and White 1991). Eye discs from third instar larvae of strong *GMR-sina* lines displayed a normal pattern of ELAV expression (data not shown), indicating that *sina* misexpression during the period of photoreceptor recruitment does not disrupt ommatidial assembly, and in particular does not cause formation of extra photoreceptor cells. In contrast, by 40 hr after pupation, signs of ommatidial disorganization were readily apparent (Figure 1, G and H). ELAV-positive cells were no longer arranged in ommatidial clusters as in wild-type,
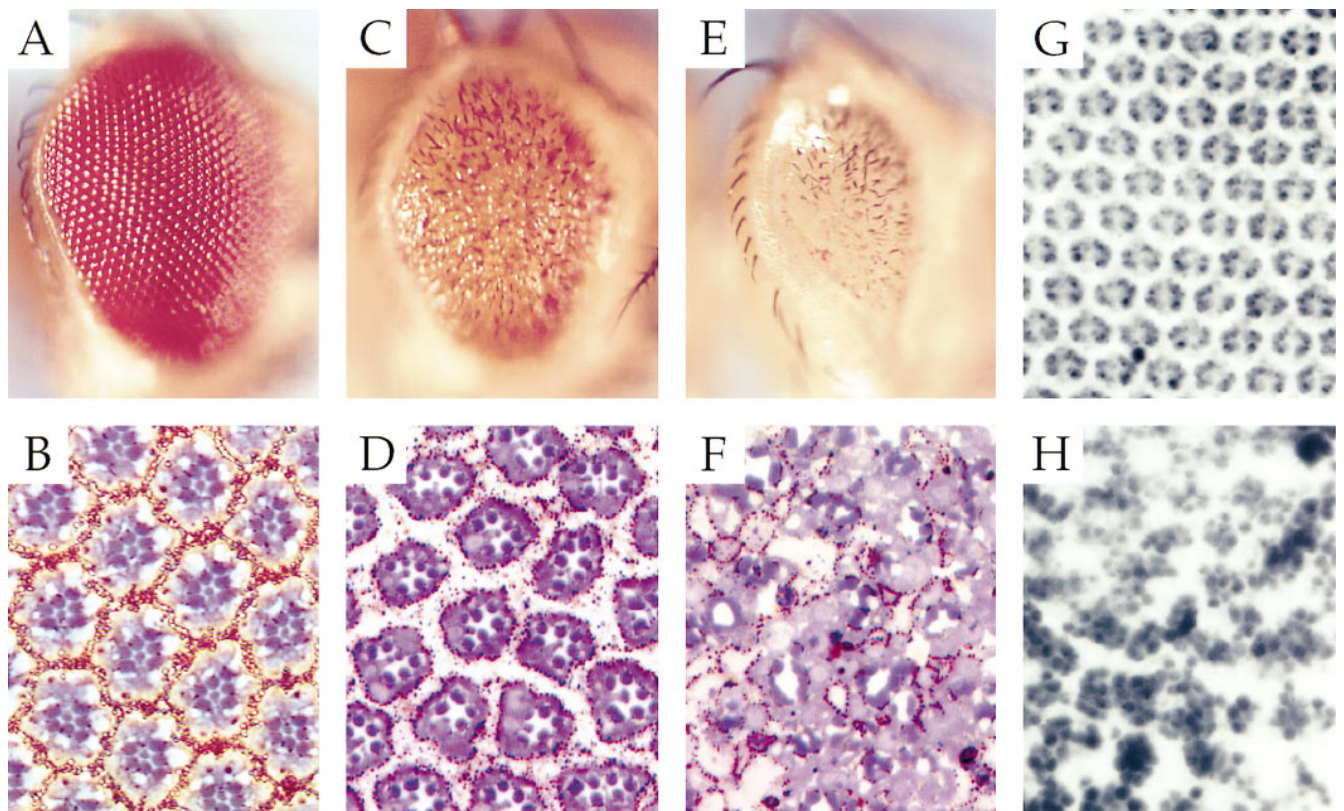

Figure 1.—*sina* misexpression phenotypes. Light photomicrographs of retinae from wild-type (A, B, G), and medium (C, D) and strong (E, F, H) *GMR-sina* lines are shown. (A, C, E) Adult eye surfaces. Note the disorganization of the lattice and loss of pigment in the transgenic eyes. (B, D, F) Tangential sections through adult eyes. *sina* misexpression disrupts the integrity of each ommatidium, but all cell types are present. (G, H) Photoreceptor nuclei in 40 hr pupal discs, marked by ELAV expression.
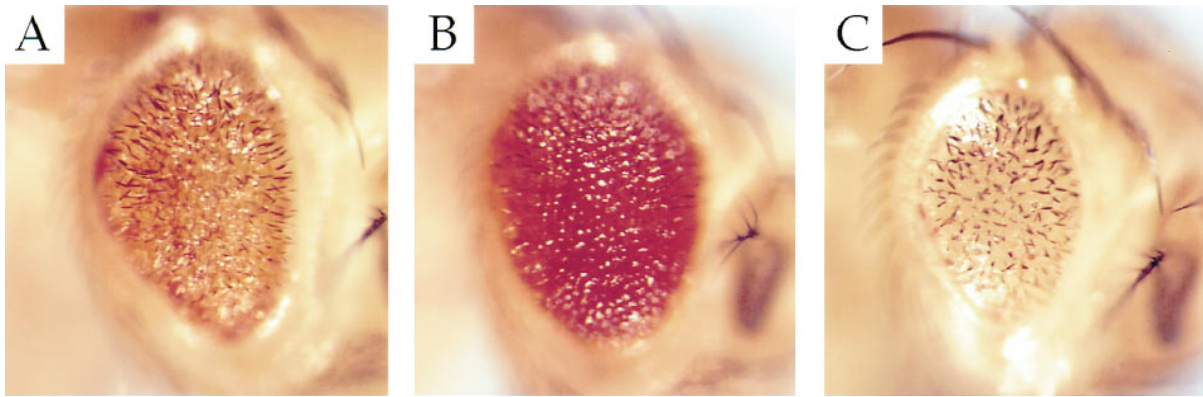
Figure 2.—Suppressors and enhancers of *GMR-sina*. Light photomicrographs of adult eyes of the following genotypes: (A) *GMR-sina/+*. (B) *GMR-sina/SS2-1*[s35] (C) *GMR-sina/ES2-1*[e1].

but instead appeared scrambled about the pupal disc. Although this disorganization prevented an accurate determination of the number of photoreceptors in each ommatidium, the overall density of ELAV-positive cells in *GMR-sina* pupal discs appeared similar to that of wild type. A similar effect was observed in eye discs stained with an antibody against the CUT protein, which specifically labels cone cell nuclei: the patterning of cone cells appeared normal in *GMR-sina* third instar eye discs, but was clearly disorganized by the 40 hr pupal stage (data not shown).

Enhancers and suppressors of *GMR-sina*: The sensitivity of the *GMR-sina* eye phenotype to dosage of the transgene (see above) suggested that *sina* activity was at a threshhold level, and might be sensitive to perturbations in genes encoding potential regulators or effectors of *sina*. To test this idea, we asked whether mutations in genes implicated in *sina* function were capable

TABLE 1

Enhancers and suppressors of *GMR-sina*

	No. of alleles	Map position	*sina*[4] interaction	Clonal phenotype
Enhancer groups				
ES1-1	11	10F7-11A7	1.5	Occasional missing R cells
ES2-1	26	51C-52F	0.8	Wild-type
ES2-2	15	39B1-2	1.0	Wild-type
ES2-3 Sin3A	2	49B3-6	3.1	Very small clones or scars
ES2-4 Star	6	21E1-2	3.4	n.d.
ES3-1	5	84B1-2	0.6	n.d.
Suppressor groups				
SS2-1	40	23C1-2	1.1	Small rhabdomeres
SS3-1	2	84B1-2	n.d.	n.d.
SS3-2 glass	11	91A1-2	2.4	n.d.
SS3-3 UbcD1	14	88D5-6	0.9	Missing R cells; scars
SS3-4 SR3-4a	24	76D3-E	1.2	Very small clones or scars
SS3-5	2	72C1-73A3	n.d.	n.d.
SS3-6	8	64C5-E1	2.0	Fallen rhabdomeres
SS3-7	2	67C4-5	2.0	Fallen rhabdomeres

n.d., not determined.
Six enhancer and eight suppressor groups were identified by lethal complementation. Not shown in the table are 73 mutants that complement all groups. Map positions were initially defined by meiotic recombination analysis of the modifier phenotype, and subsequently refined by lethal complementation tests using deficiencies and P element lines. Alleles of *ES3-1* and *SS3-1* mapped to the same location and failed to complement for recessive lethality, and thus are likely opposing alleles of the same locus. Modification of the *sina*[4] phenotype was scored in eye sections from flies heterozygous for a given modifier mutant, and homozygous for *sina*[4] (see materials and methods for details). Interaction strength was calculated as the ratio of percent R7⁻ ommatidia in *Modifier/+*; *sina*[4] eyes over percent R7⁻ ommatidia in *sina*[4] eyes. Values between 0.8 and 1.2 represent insignificant interaction. *glass-sina*[4] data is from Carthew *et al.* 1994.
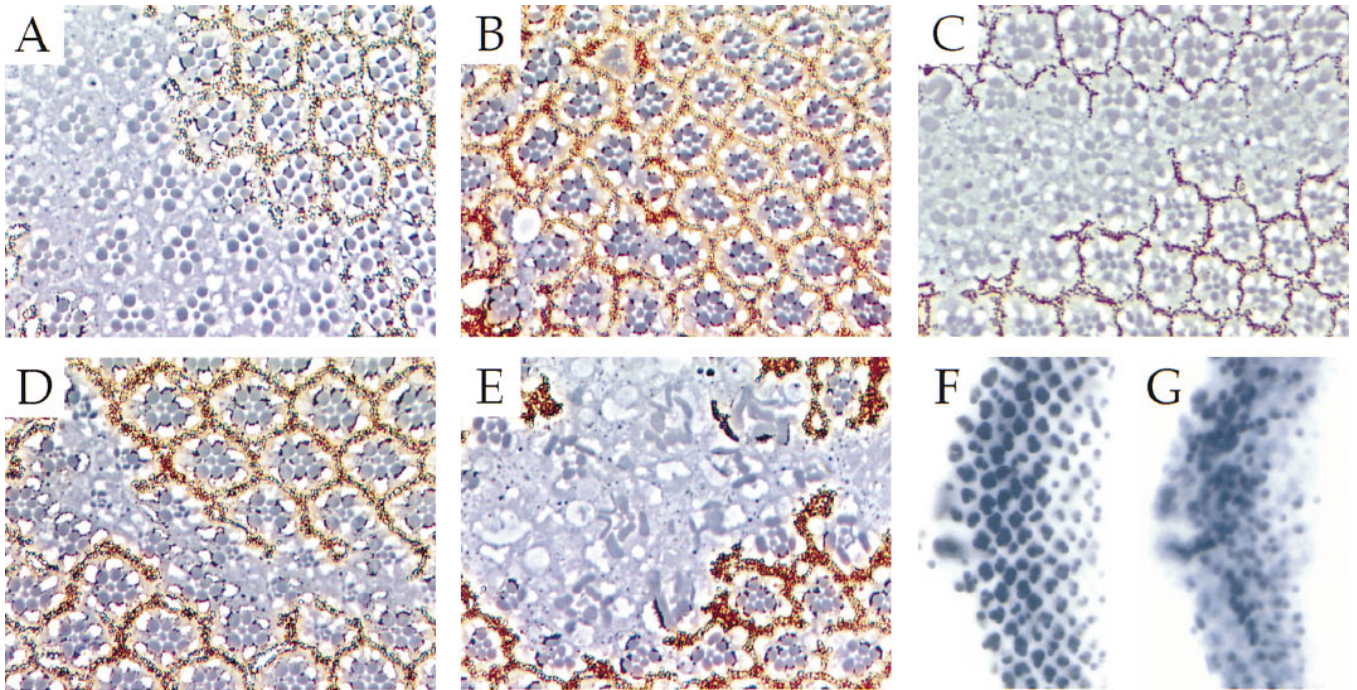
Figure 3.—Homozygous enhancer and suppressor phenotypes. Shown are tangential sections of adult eyes containing clones of cells homozygous for an enhancer or suppressor mutant. Clones are marked by the absence of pigment granules. Five phenotypic classes were observed; one example of each is shown. (A) Class 1 mutant clones result in apparently normal ommatidia. Example shown: *ES2-2^{e10}*. (B) Class 2 clones do not survive to adulthood; eyes in which such clones were generated contain large vacuoles, ommatidia with missing photoreceptor cells, and disruptions in the pigment lattice. *SS3-3^{s81}* shown. (C) Class 3 ommatidial defects include aberrant rotation and a low frequency of missing photoreceptor cells. *ES1-1^{e401}* shown. (D) Photoreceptor cells within class 4 clones bear rhabdomeres that are smaller than wild-type. *SS2-1^{s35}* shown. (E) Class 5 clones result in a loss of structural integrity of the retina; as a result rhabdomeres can be observed transversely rather than in cross section. *SS3-7^{s308}* shown. (F, G) Third instar eye disc from *SS3-7^{s308}* homozygote, stained with ELAV antiserum to identify photoreceptor nuclei. While most ELAV-positive nuclei have a normal apical position (panel F), a substantial fraction have failed to migrate and are found in the basal region of the disc (G).

of enhancing or suppressing the *GMR-sina* eye phenotype. Of nine *Enhancers of sina* previously identified in a screen for dominant modifiers of a weak *sina* allele (Carthew *et al.* 1994), three were found to modify the severity of the *GMR-sina* phenotype: *E(sina)5* and *E(sina)6* suppressed the phenotype, whereas *E(sina)2* behaved as an enhancer in this assay (data not shown). *E(sina)5* is allelic to *glass*, and probably alleviates the eye phenotype by decreasing expression of the *glass*-dependent pGMR transgene. Interestingly, *E(sina)2* enhances the eye phenotypes caused by both overexpression and loss of *sina* function. This locus was also isolated as a dominant enhancer of a temperature-sensitive allele of *sevenless* (*E[sev]3E*; Cutforth 1994). In contrast to these results, the *GMR-sina* phenotype was not modified by the Ras signaling pathway mutants *Ras1^{e1b}*, *Sos^{e4G}*, *GAP^{A13P}*, nor by strong mutations in *sina* itself (alleles C2, F1, I1, and N6; data not shown). Although limited in scope, these results suggested that the *GMR-sina* phenotype is sensitive to the dosage of at least some genes implicated in *sina* function, and that this phenotype could be used to identify mutations in additional components. We therefore decided to conduct a screen for such mutants.

Approximately 96,000 progeny of mutagenized flies were examined for their ability to dominantly modify the external eye phenotype of an intermediate strength *GMR-sina* line (see materials and methods). This screen yielded 242 dominant modifier mutants, 87 of which enhanced and 155 suppressed the eye phenotype. The strongest suppressor mutants were able to rescue *GMR-sina* eyes to a nearly wild-type appearance (Figure 2B), whereas strong enhancers increased the severity of the intermediate *GMR-sina* tester to that resembling a strong *GMR-sina* line (Figure 2C). Mutants of low to medium strength produced more subtle modifications of the *GMR-sina* phenotype (not shown). Each mutant was localized to an individual chromosome, and a corresponding balanced line was established. Tests for homozygous viability revealed that 223 (92% of total) of the lines are recessive lethal, indicating these modifier mutations either disrupt a gene required for viability, or are genetically linked to one or more such lethals. This recessive lethality was used as a basis for complementation testing to determine allelism among the mutants. Of 242 mutants, 169 (70% of total) fall into eight suppressor and six enhancer groups of 2–40 members (Table 1). Each of the remain-

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SIN3A   MMKRTRVDEV QFGTRPVPQT SGGVGVGVVG VTGGGPTSGG GGTATVGVNT TGVTIGTVVP   60

SIN3A   SAHNATISGI GSIHHRILTP QHGGAQTIAY LPSTTPTATN LKTTTSIVDS TTAGGPVGAG  120
mSin3A                                                                MKRR    4

SIN3A   AQVAVGIGSA AGGRSVVVST GSTGTQTLQY TTSYSVASIQ AGGTLKANTA DGANTVQIHV  180
mSin3A  LDDQESPVYA AQQRRIPGST EAFSHQHR.. ....VLAPAP PVYEAVSETM QSATGIQYSV   58

SIN3A   TGRRTANNPA SAQTVSSSSQ TGTIRQRTIS GTQTVATAVG NLATISQQQP VQQSPLGKAQ  240
mSin3A  ....APNYQV SAVPQSSGSH GPAI..AAVH SSHHHPTAVQ .......... ..........   92

SIN3A   TPPSSVVANS IPVGGTTPPQ GQSGNATPRL KVEDALSYLD QVKYQYADQP QIYNNFLDIM  300
mSin3A  .PHGGQVVQS HAHPAPPVAP VQGQQQFQRL KVEDALSYLD QVKLQFGSQP QVYNDFLDIM  151

                                 PAH1
SIN3A   KEFKSHCIDT PGVIERVSTL FKGHTELIYG FNMFLPPGYK IEIHSDALGC SVPVVSMPSP  360
mSin3A  KEFKSQSIDT PGVISRVSQL FKGHPDLIMG FNTFLPPGYK IEVQTNDM.. ..........  199

SIN3A   PGAPTSTGTV HMLTGNSSMS GAGHIAIKTT NAATLTPAAG AGAAAAAAAV AQIQSAGAVN  420
mSin3A  .......... .......... .......... .......... .......... ........VN  201

SIN3A   LMTHGGASLT QTTIHALQQA TPPQSQSPGG GHVHVSVTAA NAVVPGQPGI SVSAHNVPQN  480
mSin3A  VTTPGQ..VH QIPTHGIQPQ PQPPPQHPSQ P......SSQ SAPTPAQPAP QPTAAKV...  250

SIN3A   YSRDRERATI TPTGQMAGAA ANVNASASIV VGGPPTPNSL SELSPHGGAG GGPGAGAAQH  540
mSin3A  .SKPSQLQAH TPASQQTPPL P.PYASPRSP PVQPHTPVTI S.......LG TAPSL.....  296

SIN3A   NLHHIQQAHQ SILLGETGQQ NQPVEFNHAI TYVNKIKNRF QNQPAKYKKF LEILHDYQRE  600
mSin3A  .......... ........QN NQPVEFNHAI NYVNKIKNRF QGQPDIYKAF LEILHTYQKE  338

                                 PAH2
SIN3A   QKVMKEGSLN QGKMLTEQEV YTQVAKLFGQ DEDLLREFGQ FLPDATNHQS GQYMSKSASV  660
mSin3A  QRNAKEAGGN YTPALTEQEV YAQVARLFKN QEDLLSEFGQ FLPDA...NS SVLLSKTTAE  395

SIN3A   HNDHGKRPTA TLSGGAHITM SSASPAPSGS PLHLGATTLP QIDKSAHAAA IGNLSAVNTS  720
mSin3A  KVDSVRNDHG GTVKKPQLNN KPQRPSQNGC .......... QIRRHSGTGA TPPVKKKPKL  445

SIN3A   VSIKTYNNNQ QQQNHVIGSG LNATRNDILF EKDYHAGLQQ QAHQRGAGVG GHHHLAGTAA  780
mSin3A  MSLKESSMAD ASKHGVGTES L......... .......... .......... ..........  466

SIN3A   GANIGRPGVG ASVMVSYDKE HRNNHHVQKY VGHAPNQNLT HGHNAKKSPS YGIPSVIGSM  840

SIN3A   PHISDNSLDR SSPGISYATP PLPSGPHGQH NSGSATRRPG DDSLVGHYAS GAPPAKRPKP  900
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ing 73 mutants complement all groups and each other, and thus may represent single alleles of loci that were disrupted only once in this screen, or multiple alleles of one or more complementation groups with no apparent homozygous phenotype, which would not have been detected in our complementation analysis. In addition, some of these mutants may represent viable alleles of one or more of the 14 defined complementation groups.

Three of the groups represent new alleles of genes with previously described roles in eye development. As expected from our pilot screen, several new alleles of *glass* (*SS3-2*, for Suppressor of *GMR-sina*, chromosome 3, group 2) were identified as strong suppressors of *GMR-sina* (Table 1). *ES2-4* (Enhancer of *GMR-sina*, chromosome 2, group 4) represents six new alleles of *Star*, a member of the spitz group of mutants which acts in the EGF signaling pathway (Kolodkin *et al.* 1994). The 24 alleles of *SS3-4* were found to be allelic to *SR3-4a*, a gene of unknown function identified in a screen for genes that act downstream of *Ras1* during eye devel-

opment (Karim *et al.* 1996). The remaining 11 groups represent genes not previously implicated in eye development.

Interactions with a hypomorphic *sina* allele: To begin to characterize these new mutations, we tested whether they interact genetically with a partial loss of function *sina* allele, *sina⁴* (Carthew and Rubin 1990). This allele provides a reduced amount of *sina* activity, and has been useful in detecting genetic interactions between *sina* and putative regulators and effectors of *sina* (Carthew *et al.* 1994). We expected that some of the modifier mutants might act in an opposite direction in this assay; that is, that suppressors of *GMR-sina* would enhance the loss of function *sina⁴* phenotype, and *GMR-sina* enhancers would suppress *sina⁴*.

Of twelve groups tested, seven acted as dominant modifiers of *sina⁴* (Table 1). Four of these groups acted in the expected direction: *SS3-2*, *SS3-6*, and *SS3-7* enhanced *sina⁴*, and *ES3-1* behaved as a suppressor. In addition, three groups, *ES1-1*, *ES2-3*, and *ES2-4* enhanced the severity of both *GMR-sina* and the hypomorphic

PAH3

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SIN3A    YCRDVSFSEA  SSKCTISDAA  FFDKVRKALR  SPEVYDNFLR  CLTLFNQEIV  SKTELLGLVS  960
mSin3A   ..........  ..........  FFDKVRKALR  SAEAYENFLR  CLVIFNQEVI  SRAELVQLVS  506

SIN3A    PFLMKFPDLL  RWFTDFLGPP  SGQPAGGLID  GMPLAATQRQ  GGGSSNSSHD  RGTSHQSAAE  1020
mSin3A   PFLGKFPELF  NWFKNFLG..  ..........  .......YKE  SVHLESFPKE  RATE.....G  542

SIN3A    YVQDVDLSSC  KRLGASYCAL  PQSTVPKKCS  GRTALCREVL  NDKWVSFPTW  ASEDSTFVTS  1080
mSin3A   IAMEIDYASC  KRLGSSYRAL  PKSYQQPKCT  GRTPLCKEVL  NDTWVSFPSW  .SEDSTFVSS  601

SIN3A    RKTQFEETIY  RTEDERFELD  LVIEVNSATI  RVLENLQKKM  SRMSTEELSK  FHLDDHLGGT  1140
mSin3A   KKTQYEEHIY  RCEDERFELD  VVLETNLATI  RVLEAIQKKL  SRLSAEEQAK  FRLDNTLGGT  661
         .

SIN3A    SQTIHQRAIH  RIYGDKSGEI  ITGMKKNPFV  AVPIVLKRLK  VKEEEWREAQ  KTFNKQWREQ  1200
mSin3A   SEVIHRKALQ  RIYADKAADI  IDGLRKNPSI  AVPIVLKRLK  MKEEEWREAQ  RGFNKVWREQ  721

SIN3A    NEKYYLKSLD  HQAINFKPND  MKALRSKSLF  NEIETLYDER  HDQ..EDDAM  EPFGPHLVLP  1258
mSin3A   NEKYYLKSLD  HQGINFKQND  TKVLRSKSLL  NEIESIYDER  QEQATEENAG  VPVGPHLSLA  781

SIN3A    YKDKTILDDA  ANLLIHHVKR  QTGIQKQEKQ  KIKQIIRQFV  PDLFFAPRQP  LSDDERDDAF  1318
mSin3A   YEDKQILEDA  AALIIHHVKR  QTGIQKEDKY  KIKQIMHHFI  PDLLFAQRGD  LSDVEEEE..  839

SIN3A    PFLVDDNTKM  DVDSPLGRTE  SSTRNAKSTP  SESASPARSN  ASTSSVTPAG  IKKETDDSKA  1378
mSin3A   ..........  ..........  ..........  ..........  ..........  .EEEMD....  844

SIN3A    TTGSFAPASS  ATASSATPVD  DATPSTSSAA  AAASAASSST  VSGTEGKPKD  DPLSSHKEEG  1438
mSin3A   ..........  ........VD  EAT.......  ..........  .....GAPK.  ....KHNGVG  859

SIN3A    AGCTSSGVAT  SPRQAQDTAG  AGVDVEIKLE  HPADFSNPKL  LPPHAHGQRE  DESYTLFFAN  1498
mSin3A   GSPPKSKLLF  SNTAAQKLRG  M.........  ..........  ..........  DEVYNLFYVN  890
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PAH4

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SIN3A    NNWYLFLRLH  AILCDRLHVM  YERARLLAIE  EERCRVNRRE  ..........  STATALRLKP  1548
mSin3A   NNWYIFMRLH  QILCLRLLRI  CSQAERQIEE  ENREREWERE  VLGIKRDKSD  SPAIQLRLKE  950

SIN3A    KPEIQVEDYY  PTFLDMLKNV  LDGNMDSNTF  EDTMREMFGI  YAYISFTLDK  VVSNAVRQLQ  1608
mSin3A   PMDVDVEDYY  PAFLDMVRSL  LDGNIDSSQY  EDSLREMFTI  HAYIAFTMDK  LIQSIVRQLQ  1010

SIN3A    YCVTERAALD  CVELFATEQR  RGCIGGFCRD  AHKTFDREMS  YQRKAESILN  EENCFKVYIY  1668
mSin3A   HIVSDEVCVQ  VTDLYLAENN  NGAIGGQLNS  QTSRSLLESA  YQRKAEQLMS  DENCFKLMFI  1070

SIN3A    KIDCRV..TI  ELLDSEPEEV  DKPAALKAQK  FSKYVERLAN  PALGGGGNTG  RSDSALGNDS  1726
mSin3A   QSQGQVQLTV  ELLDTEEENS  DDP..VEAER  WSDYVERYMS  SDTTSPELRE  HLAQKPVFLP  1128

SIN3A    VVDGSDIKTE  ADEDTAEVNT  KA*                                          1748
mSin3A   RNLRRIRKCQ  RGREQQEKEG  KEGNSKKTME  NVESLDKLEC  RFKLNSYKMV  YVIKSEDYMY  1188

mSin3A   RRTALLRAHQ  SHERVSQGKS  RGIHNFFLQI  L*                              1219
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Figure 4.—Amino acid sequence comparison of the deduced Drosophila SIN3A and mouse mSin3A proteins. Identical residues are highlighted. Gaps introduced by the alignment algorithm are indicated by dots. The four boxed regions represent the PAH motifs of mSin3A (Ayer *et al.* 1995). Seven cDNAs that encode the shown Drosophila SIN3A sequence were identified. In addition, a single cDNA representing an alternatively spliced transcript was identified, which encodes a protein whose extreme carboxy-terminus differs from that of the sequence shown. The two sequences are identical through residue #1743, at which point the alternative sequence diverges to read: HTQSQQWTLL ESENEKYIWS SPLKIHVQSY*. The GenBank accession number of these cDNA sequences are AF024603 and AF024604.

sina⁴ allele. The five remaining groups had little or no effect on the severity of *sina⁴*, suggesting the dosage of these genes is limiting when an excess of SINA protein is present, but apparently not when *sina* activity is reduced.

Homozygous mutant phenotypes: To test more directly the roles of the modifier groups in eye development, we examined eye phenotypes caused by homozygous loss of these loci. Since all of the groups except *SS3-2 (glass)* reside on recessive lethal chromosomes,

clones of cells homozygous for these mutations were generated by mitotic recombination using the FLP-FRT technique (Xu and Rubin 1993). Five classes of adult eye phenotypes were observed (Table 1). Two groups, *ES2-1* and *ES2-2*, gave rise to large clones of wild-type appearance, and thus appear to be dispensable for normal eye development (Figure 3A). A second class (*ES2-3*, *SS3-4*, and most *SS3-3* alleles) did not give rise to clones, but instead generated scars across the eye. Sections through such scars revealed missing photorecep-

tor and pigment cells, and the presence of large vacuoles (Figure 3B). These groups most likely affect cell survival or proliferation. *ES1-1* and three weak alleles of *SS3-3* comprise a third phenotypic class (Figure 3C), producing clones of nearly normal size, with occasional reductions in the number of photoreceptors per ommatidia. Two final classes were assigned based on defects in the morphology of cells within clones. Photoreceptors within *SS2-1* clones (class 4) were found to bear extremely small rhabdomeres (Figure 3D). The mutant rhabdomeres were smaller than wild-type R7 rhabdomeres, indicating that they probably are not the result of a transformation to the R7 fate. The overall smaller cell size resulted in a distortion of the regular lattice across the clone. A similar phenotype has been observed in *E(sina)4* homozygous mutant clones (Carthew *et al.* 1994). Finally, photoreceptor cells within clones of *SS3-6* and *SS3-7* mutants (class 5) also displayed rhabdomeres with abnormal morphology. In such clones, rhabdomeres were often projected tangentially in the retinal plane, rather than radially as in wildtype (Figure 3E). This collapsed rhabdomere phenotype is similar to that described for the *marbles* mutant (Fischer-Vize and Mosley 1994). A *marbles*-like phenotype was also observed in developing eye discs of *SS3-7* homozygotes (which survive until pupation). Photoreceptor nuclei in *SS3-7* mutants often failed to migrate to the apical surface of the disc (Figure 3, F and G), as has been described for *marbles* (Fischer-Vize and Mosley 1994).

Molecular analysis: Mapping by meiotic recombination and deficiency analysis revealed that several of the *GMR-sina* modifier groups are located near the sites of lethal P-element insertions maintained as part of the Berkeley Drosophila Genome Project (Spradling *et al.* 1995). Complementation tests between these lines and the modifier groups identified five groups allelic to one or more lethal P lines, providing a starting point for the molecular cloning of these loci. We report here the molecular characterization of *SS3-3* and *ES2-3*.

The *SS3-3* group failed to complement four lethal P element lines in the 88D5-6 region. Sequence analysis of cDNAs spanning the insertion sites of two such lines (see materials and methods) identified this locus as the *UbcD1* gene, which encodes a ubiquitin conjugating enzyme involved in selective protein degradation (Treier *et al.* 1992). Male-sterile alleles of this locus have recently been identified and are known as *effete* (Cenci *et al.* 1997).

Two P lines at 49B3-6 were found to be allelic to the ES2-3 group. The transcription unit disrupted by these P insertions (see materials and methods) is predicted to encode a polypeptide sequence with high similarity to members of the Sin3 family of transcriptional repressors (Wang *et al.* 1990; Ayer *et al.* 1995). The protein sequence in the database most closely related to the deduced *ES2-3* sequence is that of mouse mSin3A, and we have therefore renamed the *ES2-3* locus *Sin3A*. Figure 4 shows an alignment of the fly and mouse SIN3A sequences. The two proteins share 33% amino acid identity overall, with the highest level of sequence conservation centered on the four paired amphipathic helix (PAH) domains and the region between PAH3 and PAH4, which is required for association of mSin3A with histone deacetylase enzymes (Laherty *et al.* 1997). In addition, the Drosophila sequence is longer by 529 amino acids than the mouse, due to a longer amino-terminal tail and insertions between the PAH domains.

DISCUSSION

Expression of *sina* at high levels in all cells of the eye disc causes a severe disruption of the structure and organization of the adult eye. This disruption is a relatively late event, occurring after the initial period of ommatidial assembly and photoreceptor cell fate specification. Given the requirement for *sina* in R7 photoreceptor development, a reasonable expectation was that ectopic *sina* expression might cause transformation of cone cell precursors into R7 photoreceptors, as observed for ectopic expression of *boss* (Van Vactor *et al.* 1991), *phyllopod* (Chang *et al.* 1994), and activated *Ras1* (Fortini *et al.* 1992). However, this appears not to be the case for *sina*, as no evidence of supernumerary photoreceptor cell formation was observed in *GMR-sina* flies. Since defects in retinal structure were first observed during the pupal stage, a time at which SINA is normally expressed in all cells in the eye disc (Carthew and Rubin 1990), we conclude that the defects observed in *GMR-sina* flies are most likely caused by higher than normal levels of SINA protein in these cells, rather than by inappropriate timing or patterning of SINA expression.

The relatively late manifestation of the *GMR-sina* phenotype suggests that cells in the eye disc remain responsive to *sina* activity beyond the time at which the R7 cell is normally recruited. A requirement for *sina* during these later stages of eye development has been described previously for a subset of R1-6 photoreceptors, which in strong *sina* mutants initially appear to be properly specified, but subsequently can lose their normal photoreceptor morphology during pupation (Carthew and Rubin 1990). Moreover, loss of function mutations in the *ttk* gene result in formation of supernumerary R7 cells during the pupal stage (Lai *et al.* 1996). We and others have recently demonstrated that *ttk* interacts physically and genetically with *sina,* and that *sina* is required for degradation of TTK in response to activation of the RAS-MAPK pathway (Tang *et al.* 1997; Li *et al.* 1997; and see below). Taken together, these results suggest that some cell types in the developing eye may require continuous inductive signals to maintain their particular developmental program, and

that these signals are dependent upon proper levels of SINA protein. Alternatively, *sina* may have other functions during the later stages of eye development.

A major goal of this study was to identify genes involved in regulating and effecting *sina* activity. Among our collection of 14 *GMR-sina* enhancer and suppressor groups are such candidate genes. Of the three groups with known roles in eye development, *SS2-2 (glass)*, *ES2-4 (Star)*, and *SS3-4 (SR3-4)*, two appear to interact nonspecifically with *GMR-sina*. Although genetic interaction between *sina* and *glass* was described in a previous report (Carthew *et al.* 1994), the primary suppressive effect of *glass* mutants on the *GMR-sina* phenotype is most likely due to reduced expression of the *glass*-dependent transgene. Additionally, like previously existing *Star* alleles, *ES2-4* mutants have a dominant rough eye phenotype independent of *GMR-sina*, and thus the interaction observed between *Star* and *GMR-sina* appears to reflect independent additive effects of each mutation.

Mutations allelic to *SS3-4* have been isolated previously as dominant suppressors of a rough eye phenotype caused by an activated *Ras1* transgene (Karim *et al.* 1996). *sina* has been shown to be required for the transformation of cone cells to photoreceptors caused by activated *Ras1* (Fortini *et al.* 1992), a requirement we attribute to SINA's role in degrading TTK. Characterization of mutants that interact with both *sina* and *Ras1* may lead to a further understanding of the relationship of these genes. Two additional suppressor groups (*SS2-1* and *SS3-3*; data not shown) also tested positive for interactions with activated *Ras1*, as did several mutants isolated in a previous screen for enhancers of *sina* (Carthew *et al.* 1994).

Molecular cloning of the remaining genes identified in this screen has begun to provide additional insight into the mechanisms of SINA function. The finding that *SS3-3* encodes the ubiquitin conjugating enzyme UBCD1 is consistent with a role for SINA in ubiquitin-dependent processes, a possibility initially suggested by interactions between *sina* and *fat facets*, which encodes a deubiquitinating enzyme (Carthew *et al.* 1994; Huang *et al.* 1995) Recent experiments have found that SINA-dependent degradation of TTK in cell culture probably occurs via the ubiquitin-dependent proteolysis pathway (Li *et al.* 1997). The genetic interaction between *sina* and *UbcD1* presented here, as well as our demonstration of physical interaction between SINA and UBCD1 (Tang *et al.* 1997), provides a molecular framework for beginning to understand how SINA may regulate the stability of proteins such as TTK.

Members of the Sin3 class of transcriptional corepressors serve as requisite components of the Mad-Max repressor complex (Ayer *et al.* 1995). In this report, we have demonstrated that a Drosophila member of this family, *Sin3A*, interacts genetically with *sina*. At present, the role played by *Sin3A* during development is unclear. We were unable to recover clones of cells homozygous for *Sin3A* mutations, suggesting that this gene is required for cell proliferation or survival. Identification of mutations in Drosophila *Sin3A* should contribute to our understanding of this important class of transcriptional regulators. Elucidation of the roles played by the products of the remaining genes identified in this screen awaits their further characterization.

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