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Undifferentiated cells in the developing *Drosophila* eye influence facet assembly and require the Fat facets ubiquitin-specific protease

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SUMMARY

The *Drosophila* compound eye develops by a complex series of cell interactions where multiple positive and inhibitory cues guide cells in each facet into their positions and fates. The results of many genetic and molecular experiments have led to the view that facet assembly is directed by cells within developing ommatidial preclusters. Here *fat facets* mutants and the cloned *fat facets* gene were used to show that, in order to limit the number of photoreceptors in a facet to eight, undifferentiated cells surrounding assembling facets send an inhibitory signal to extraneous cells within the facet preclusters. Generation of the inhibitory signal requires the ubiquitin-specific protease encoded by the *fat facets* gene and is thus regulated by ubiquitin-dependent proteolysis.

Key words: eye, *fat facets*, *Drosophila*, ubiquitin, Ubp

INTRODUCTION

Cells in the developing *Drosophila* eye interpret a multitude of positive and negative signals from neighboring cells in order to learn their positions and fates. The compound eye is made up of approximately 800 identical unit eyes, called facets or ommatidia, each of which is composed of 8 photoreceptors (R1-R8) surrounded by a hexagonal lattice of pigment cells and capped by four cone cells (reviewed in Wolff and Ready, 1993). The first event in ommatidial assembly is the singling out of the initial photoreceptor, R8, from a field of competent cells (Jarman et al., 1994) by inhibitory cell interactions involving the *Notch* signaling pathway and the *scabrous* gene (Cagan and Ready, 1989; Baker et al., 1990; Mlodzik et al., 1990; Ellis et al., 1994; Parks et al., 1995; Baker and Zitron, 1995; Lee et al., 1996). The R8 cell is then thought to initiate a series of local cell inductions which recruit the appropriate number of photoreceptor cells into each facet (see Fig. 1). First, R8 is thought to signal the adjacent R2 and R5 precursor cells by secreting Spitz protein, a TGFα homolog (Rutledge et al., 1992) likely to be the ligand for the *Drosophila* EGF receptor (DER) which is present on the surfaces of all photoreceptor cell precursors (Tio et al., 1994; Freeman, 1994; Schweitzer et al., 1995a). The R2/5 pair probably secrete Spitz also, and thus contribute to the signaling of the adjacent R3/4 and then R1/6 precursors by R8 (Freeman, 1994; Tio et al., 1994). One of the cellular responses to DER activation is the production of Argos (Golembo et al., 1996), a secreted inhibitor of DER which prevents DER activation in cells other than the photoreceptor precursors adjacent to R8 and R2/5 (Schweitzer et al., 1995b). The best understood induction event is the recruitment of R7, the final photoreceptor cell to join the facet. The Sevenless receptor on the R7 cell surface is activated by its ligand, Bride-of-sevenless, expressed on the surface of R8 (reviewed in Dickson and Hafen, 1993). Thus, once R8 is specified, all positive and negative signals for photoreceptor recruitment appear to emanate from determined cells within the facet.

The *fat facets (faf)* gene product is necessary in order for the so-called 'mystery cells' (Tomlinson and Ready, 1987), which are transiently associated with the early 7-cell facet precluster (Fig. 1), to leave the preclusters; the mystery cells become ectopic R3/4-like photoreceptors in *faf* mutant eyes (Fischer-Vize et al., 1992a). Often, *faf* mutant facets also contain ectopic R7-like cells of undetermined origins (Fischer-Vize et al., 1992a; Huang et al., 1995). The *faf* gene encodes a ubiquitin-specific protease (Ubp) (Huang et al., 1995), one member of a large family of enzymes first characterized in yeast (Tobias and Varshavsky, 1991; Baker et al., 1992; Papa and Hochstrasser, 1993; Hochstrasser, 1995). Ubiquitin (Ub) is a highly conserved polypeptide found in all eukaryotes, the major function of which is to target proteins for complete or partial degradation by a multisubunit complex called the proteasome (reviewed in Ciechanover, 1994; Hochstrasser, 1995). Ubps, which cleave Ub-protein bonds, play a variety of roles in Ub-mediated protein degradation (Baker et al., 1992; Papa and Hochstrasser, 1993; Hochstrasser, 1995). Faf appears to be a regulatory Ubp that removes Ub from perhaps a specific ubiquitinated substrate, thereby preventing the degradation or processing by the proteasome of an unidentified protein (Huang et al., 1995).

The *faf* gene is expressed almost ubiquitously in the larval eye disc, in cells within and outside the facet (Fischer-Vize et al., 1992a). Analysis of facets mosaic for *faf*+ and *faf*– photoreceptor cells suggests that *faf* is not required by the mystery cells nor in any particular photoreceptor cells within the facet (Fischer-Vize et al., 1992a). However, the results of the mosaic analysis are also consistent with a role for *faf* in positively regulating the activity of an inhibitor of photoreceptor fate

secreted by cells within the facet, like Argos. In addition, the ectopic R7-like cells in *faf* mutant facets could be cone cell precursors which normally require two different proteins to prevent them from becoming ectopic R7 cells: the DNA-binding protein Yan (Lai and Rubin, 1992; O'Neill et al., 1994; Treier et al., 1995) and Gap1, a negative regulator of Ras activation (Gaul et al., 1992; Rogge et al., 1992). The mosaic experiments do not rule out a role for *faf* in positively regulating Yan or Gap1 activities within cone cell precursors.

Using hybrid gene constructs to express *faf* in specific subsets of cells in the developing eye, here it is demonstrated that the *faf* gene is essential only early in eye development and only outside of the 7-cell precluster and cone cell precursors. Thus, the critical role of *faf* in eye development is not in the regulation of Argos, Yan or Gap1 activities, nor in the regulation of any other protein that functions within cells of the developing facets. Moreover, the results presented show that undifferentiated cells outside the facet precluster direct ommatidial assembly by sending to the mystery cells and other cells an inhibitory signal, regulated by Faf protein.

MATERIALS AND METHODS

Drosophila strains

All flies were kept on standard food at 25°C unless otherwise noted. Mutant *faf* alleles are described in Fischer-Vize et al. (1992a).

Gal4 insertions and *UAS* reporters

All *Gal4* insertions and reporters are *P[w+]*. The *Gal4* insertion in *elav* (Rebay and Rubin, 1995) was obtained from T. Laverty. Two different *Gal4* insertions in *scabrous (sca)* were used; *T3* (Kramer et al., 1995) was obtained from Y. Hiromi and sca^{4512} (M. Mlodzik, unpublished) from M. Mlodzik. Two independent *Gal4* insertions in the *hairy (h)* locus were used; *1J3* (Brand and Perrimon, 1993) was obtained from N. Perrimon and *H10* (Y. Hiromi, unpublished) from Y. Hiromi. The *UAS-lacZ* reporter strain used (Brand and Perrimon, 1993) is a second chromosome insertion obtained from A. Brand that expresses cytoplasmic β-galactosidase and the *UAS-nuclacZ* strain (*UAS-NZ20b;* Y. Hiromi, unpublished) is a second chromosome insertion that expresses nuclear β-galactosidase and was a gift of Y. Hiromi.

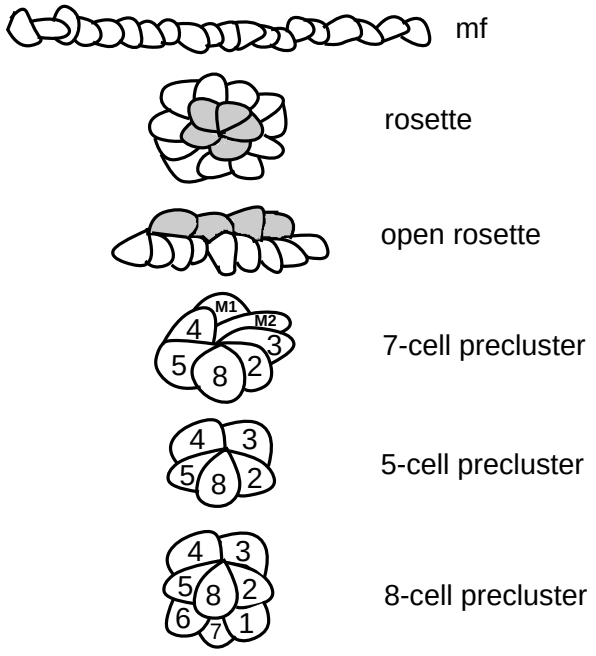


Fig. 1. Assembling facet structures early in eye imaginal disc development. Shown are structures observed at the apical surface of the eye disc posterior to the morphogenetic furrow (mf) (Tomlinson and Ready, 1987; Wolff and Ready, 1991). M1, M2 are the mystery cells and the numbers refer to photoreceptor cells R1-R8.

Suppression of *faf* by *h-Gal4*

Surprisingly, both *Gal4* insertions in the *h* locus (*1J3* and *H10*) behave as partial suppressors of faf^{BX4} in the absence of *UAS-faf*. Both insertions are known to drive expression of reporter constructs in a wide stripe of cells ahead of the morphogenetic furrow in a wild-type background (Ellis et al., 1994; Y. Hiromi, personal communication) and the endogenous Hairy protein is expressed normally in a *faf* mutant background (see below). Although both *1J3* and *H10* cause weak *h* mutations (Brand and Perrimon, 1993; Y. Hiromi, personal communication), the suppression effect is unlikely to be due to *h* mutation: three different *h* mutations, one weak (h^2) and two strong (h^{41} and *l(3)08247*; all obtained from the Bloomington Stock Center), were tested for genetic interactions with *faf* and none behave as suppressors. Thus the suppression appears to be due to the presence of Gal4 protein in the cells ahead of the furrow. As the *h* expression pattern ahead of the furrow is the region of strongest endogenous *faf* expression (Fischer-Vize et al., 1992a) and the experiments described in this paper show that this is where endogenous *faf* expression is most likely to be critical, it would be expected that *faf* expression in the *h* pattern would rescue the *faf* phenotype, at least partially. However, it was difficult to determine whether or not additional suppression of faf^{BX4} occurred in the presence of *UAS-faf* insertions because the suppression of the *faf* mutant phenotype by *h-Gal4* alone was very strong. Also, it is possible that the presence of Gal4 in these cells somehow changes the cellular physiology such that the addition of Faf protein no longer has any effect. Suppression was not observed with any of the other *Gal4* insertion lines used, none of which, in combination with *UAS-faf*, complement *faf* mutations even partially. Thus, the observation that *h-Gal4* suppresses *faf* may indicate that the *h* promoter is indeed expressing Gal4 in the cells in which Faf functions.

Promoter-*lacZ* reporters

The following strains used contain promoter-*lacZ* reporter genes where the promoters are essentially identical to those in the promoter-*faf* constructs: *ro-lacZ* (Heberlein et al., 1994; obtained from U. Heberlein), *sev-lacZ* (*P[ry+, ES3-lacZ]1;* R. Carthew, personal communication; obtained from T. Laverty), *glrl-lacZ* (construct *C* in Moses and Rubin, 1991; obtained from J. Treisman) and *glrs-lacZ* (construct *29-1* in Ellis et al., 1993; obtained from P. Garrity). The *glrs-lacZ* construct above was used in the histochemical staining experiments. For simplicity of strain construction, a slightly different *glrs-lacZ* line that contains three rather than five copies of the short *glass* oligonucleotide (construct *29-2* in Ellis et al., 1993; obtained from P. Garrity) was used in the antibody staining experiments.

Mutations tested for modification of *faf*

The following mutant alleles were tested for dominant enhancement or suppression of the *faf* mutant phenotype as described in the text. The individuals from whom the flies were obtained or references describing the alleles are indicated in parentheses. *Notch* pathway and related genes: N^{5419}, $Su(H)^{T4}$, $S(H)^{1B115}$, dx^1, mam^{1L115}, bib^1, vg^{BG}, wg^{JG} (M.Fortini), $E(spl)^{BX22}$ (A. Preiss), zw^{3M11-1} (N. Perrimon), sno^{93i} (A. Majumdar), pcx^1, *fs(1)amx* (A. Mahowald), sca^{BP2} (N. Baker), gro^{BFP2} (Fischer-Vize et al., 1992b), Ras pathway (M. Simon, M. Freeman, T. Laverty): $ras1^{e1B}$, drk^{EOA}, Sos^{e4G}, $Dsor1^{S1221}$, rl^{S352}, raf^{1129}, Gap^{1PB}. DER signaling (M. Freeman): spi^{T25}, spi^{A14},

DER^{IK35}, *DER*^{1PO2}, *aos*^{W11}, *rho*^{PD5}. Cell cycle: *Df (2R)59AB (cycB), l(3)183 (cycA), rux*^{8} (B. Thomas), *cdc*^{E10} (N. Yakubovich). Other genes (T. Laverty): *sina*^{A16}, *yan*^{1}.

Plasmid constructions

Vectors convenient for expressing any gene in the *sevenless (sev)* or *rough (ro)* patterns, under UAS control, or under the control of either 'long' or 'short' *glass (gl)* responsive elements were constructed with a unique *Asc*I site for DNA insertion as described below. The *Asc*I fragment containing a Myc-tagged *faf* cDNA and the *heat-shock protein 70 (hsp70)* expression vector are described in Huang et al. (1995). DNA manipulations were performed using standard procedures (Sambrook et al., 1989).

The short *gl*-binding site vector (pGLRS-faf) was constructed by changing the *Kpn*I site in Bluescript (Stratagene) to *Not*I and the *Bam*HI site to *Asc*I to generate pBSKNBA. A 150 bp *Xba*I fragment of pβn27.1 (Riddihough and Ish-Horowicz, 1991) containing the TATA box and transcription start of *hsp27* was cloned into the *Spe*I site of pBSKNBA to generate pBShsp27. A 200 bp *Xba*I fragment of pHZ50PL40/41 (Ellis et al., 1993; obtained from M. Ellis) containing five short *gl*-binding sites was cloned into the *Xba*I site of pBShsp27 to generate pEGhsp27/BSKNBA. An *Asc*I fragment containing the *faf* cDNA was cloned into the *Asc*I site of pEGhsp27/BSKNBA. Finally, a *Not*I fragment containing the short *glass*-binding site-*hs-faf* gene was cloned into the *Not*I site of pCasper3pA, a derivative of Casper3 (Thummel and Pirrotta, 1992; obtained from V. Pirrotta) with an 850 bp *Xba*I-*Eco*RI fragment from pC4βgal (Thummel et al., 1988) containing SV40 transcription termination sequences cloned into the *Hpa*I site.

The long *gl*-binding site vector (pGLRL-faf) was constructed by cloning a 250 bp *Xba*I fragment of pwnβE/KM1/2 (Ellis et al., 1993; obtained from M. Ellis) containing five long *gl*-binding sites into the *Xba*I site of pBShsp27 to generate pEGLhsp27/BSKNBA. The *faf* cDNA within an *Asc*I fragment was then cloned into the *Asc*I site of this vector to generate pBSEGL*hsp-faf*, and a *Not*I fragment containing the long *glass*-binding site-*hs-faf* gene was cloned into the *Not*I site of pCasper3pA.

The *sev* vector (pSEV-faf) was constructed by changing the *Xba*I site of pSP/HSS (Bowtell et al., 1989; obtained from M. Ellis) containing the *sev* promoter, to *Asc*I to generate pSP/HSS-XA, inserting the 850 bp SV40 fragment into the *Bam*HI site and the *faf* cDNA within an *Asc*I fragment into the *Asc*I site. The *sev-faf* gene was then cloned as a *Not*I fragment into the *Not*I site of the transformation vector pES/W8 (obtained from M. Ellis), which contains three *sev* transcriptional enhancers.

The UAS vector (pUAST-XA) was constructed by changing the *Xho*I site of pUAST (Brand and Perrimon, 1993; obtained from A. Brand) to *Asc*I.

The *ro* vector (pRO-faf) was constructed by cloning a 2.7 kb *Not*I fragment of pTHZ50/5′D#24 (Heberlein et al., 1994; obtained from U. Heberlein) containing the *ro* gene enhancer upstream of a *Not*I fragment from phspcDNA, which contains the *hs-faf* gene (Huang et al., 1995), into the *Not*I site of Casper3.

P element transformation

Plasmids used for injections were purified on CsCl-EtBr equilibrium density gradients (Sambrook et al., 1989). *w*^{1118} embryos were coinjected with P element plasmids and helper plasmid according to standard procedures (Spradling, 1986). P element insertions were mapped to a chromosome and stocks established using standard techniques. All transformant lines analyzed were obtained directly from injected embryos except for 15 of the 17 *ro-faf* lines and 6 of the 7 *sev-faf* lines which were obtained by mobilization of original P insertions using standard techniques. P element transformants were crossed into a *faf*^{BX4} background using standard techniques.

Analysis of adult eyes

Scanning electron microscopy (Huang et al., 1995) and fixation, embedding and sectioning of eyes for the light microscope (Tomlinson and Ready, 1987; Fischer-Vize et al., 1992a) were performed as described previously. Eye sections were photographed using a Zeiss Axioplan microscope.

Staining eye discs for β-galactosidase activity

In order to approximate the relative levels of expression of the various promoters, larval eye discs of *ro-lacZ, sev-lacZ, glrs-lacZ, glrl-lacZ* and *sca-Gal4; UAS-lacZ* were stained histochemically for β-galactosidase activity as described previously (Fischer-Vize et al., 1992a). Staining developed nearly immediately in the *glrs-lacZ* and *sca-Gal4; UAS-lacZ* discs. Staining began to develop in discs from all of the other lines after a few minutes.

Staining eye discs with antibodies

Staining with mAbRough (a gift of U. Heberlein) and mAbHairy (a gift of N. Brown) was performed as described in Fischer-Vize et al. (1992a) for mAbRough. mAbβgal (Promega) and mAbSevenless (42G11 or 36D7; a gift of M. Simon) were used as described in Fischer-Vize et al. (1992a) for mAbβgal, except that the secondary antibody used was HRP-conjugated horse-anti-mouse (Vector) and for staining *glrs-lacZ* and *sev-lacZ* discs, Co and Ni were omitted from the developing solution. Instead, after developing, discs were treated with 2% OsO4 in 0.1 M sodium phosphate for 5 minutes on ice, rinsed with 0.1 M sodium phosphate and then dehydrated with ethanol (30/peripodial membrane removed/50/100/100). Antibody-stained discs were photographed with a Zeiss Axioplan microscope.

Heat-shock experiments

In order to test for a dominant effect of *hs-faf*, otherwise wild-type larvae containing two copies of the *hs-faf* gene were subjected to a wide variety of heat-shock regimens. Embryos were allowed to develop for 4 days (to the early third instar larval stage) and then they were subjected to 30 minute-2 hour heat shocks at 37°C followed by 30 minute-4 hour recovery times 2-5 times daily for 2-5 days.

In order to test how early a heat shock could result in rescue of the *faf* mutant rough eye phenotype, *hs-faf ; faf*^{BX4}*/TM6B* flies were transferred to new food vials every 24 hours for 7 days. Eight vials containing animals at different stages of development were heat shocked simultaneously by immersion in a 37°C water bath for 1 hour and then allowed to continue development at 25°C. Thus, flies were heat shocked as embryos (0-24 hours after egg laying (hours AEL)), first instar larvae (24-48 hours AEL), second instar larvae (48-72 hours AEL), early-mid third instar larvae (72-96 hours AEL), mid-late third instar larvae (96-120 hours AEL) and as pupae. No rescue of the *faf*^{BX4} eye phenotype was ever observed in *hs-faf; faf*^{BX4} adults that had been heat shocked as embryos or first instar larvae (0-48 hours AEL). Approximately 1/5 of the flies heat shocked at 48-72 hours AEL had ~6-12 rows of wild-type facets in the posterior of their eyes. All flies heat shocked at 72-96 hours AEL showed some rescue and some flies had nearly completely wild-type eyes, except for ~6 rows at the anterior of the eye. Similar results were obtained with flies heat shocked at 96-120 hours AEL, except some eyes with a few rows of anterior or posterior roughness were observed. Pupal heat shocks produced no rescue.

The heat-shock experiments argue that Faf protein is stable for less than 56 hours. There are approximately 32 rows of facets in the eye disc and as each row requires ~2 hours to form, it takes ~64 hours for the furrow to move through all the rows. The furrow starts to move at about 12 hours into L3, or about 84 hours AEL (Tio et al., 1995). Thus, to rescue all but a few rows of the eye, or about 28 rows, Faf needs to be stable for 56 hours. If Faf is stable for 56 hours, a heat shock at 36 hours before the furrow starts to move (48 hours AEL), should result in the rescue of ~10 rows of facets. No rescue was ever seen in flies heat shocked at 24-48 hours AEL. Moreover, only 1/5 of the flies heat shocked at the next time point (48-72 hours AEL)

Table 1. Complementation of *faf*^{BX4} by promoter-*faf* expression constructs in P element transformants

Promoter-*faf* construct	Expression pattern	Number of transformant lines that rescue *faf*^{BX4} eye phenotype		
		Not at all	Partially	Nearly completely
hs-faf	Ubiquitous[1]			4
ro-faf	In furrow and R2/5, R3/4[2]		3	14
glrs-faf	Posterior edge of furrow and all cells posterior to furrow[3]	1	6	1
glrl-faf	All R-cells posterior to furrow[4]	13		
sev-faf	M1, M2, R3/4, R1/6, R7, CC[5]	7		
sca-Gal4; UAS-faf	Precluster cells at posterior edge of furrow and R8 posterior to furrow[6]	7		
elav-Gal4; UAS-faf	All R-cells posterior to furrow[7]	7		
h-Gal; UAS-faf	Stripe of cells anterior to furrow[8]		*	

All transformant lines were tested with two copies of the P element except that there was only one copy each of the *Gal4* and *UAS-faf* inserts. The structures of the promoter constructs are described in Fig. 2. Representative external eyes and retinas are shown in Figs 3 and 5.

[1]Pelham, 1982; Lis et al., 1983.

[2]Kimmel et al., 1990; Heberlein et al., 1994.

[3,4]Moses and Rubin, 1991; Ellis et al., 1993.

[5]Tomlinson et al., 1987; Bowtell et al., 1989.

[6]Baker et al., 1990; Mlodzik et al., 1990; Two independent *sca-Gal4* lines were tested (see Materials and Methods and Fig. 4).

[7]Bier et al., 1988; Rebay and Rubin, 1995.

[8]Brown et al., 1991; Ellis et al., 1994; see Materials and Methods.

*see Materials and Methods.

showed any rescue of the rough eye and the rescue was only about 5 rows. Presumably the flies with partially rescued eyes were closer to 72 hours than 48 hours old at the time of heat shock.

Protein analysis

Procedures for heat shocking embryos, generating and quantitating embryo protein extracts and Western blotting and developing were exactly as described in Huang et al. (1995). The Coomassie-stained gel was generated using standard procedures (Sambrook et al, 1989).

RESULTS

faf function is essential only outside the facet precluster

In order to determine which cells in the developing eye require *faf* expression, hybrid genes that express *faf* in different groups of cells were generated and tested for their ability to complement *faf* mutations. Expression of the *faf* cDNA in different patterns in the eye disc was achieved either by construction of promoter-*faf* gene fusions or by controlling the expression of a *UAS-faf* construct with different *Gal4* enhancer trap lines (Table 1; Fig. 2). Many P element transformant lines containing each construct were generated and crossed into a *faf* null mutant *(faf*^{BX4}*)* background to test the ability of the construct to rescue the *faf* mutant eye phenotype to wild-type. It was observed that the three constructs resulting in at least partial rescue of the *faf* mutant phenotype have two common characteristics, not shared by the non-rescuing constructs: they express *faf* early in eye development and outside the cells within the developing facets (Table 1; Figs 2, 3).

Four constructs, *ro-faf, glrs-faf, sev-faf* and *sca-Gal4; UAS-faf,* are key to the conclusion that *faf* is required early and outside the cells within the facet. Both constructs that can substitute at least partially for the endogenous *faf* gene, *ro-faf* and *glrs-faf,* are expressed ubiquitously early in eye development before the mystery cells normally leave the preclusters. In contrast, *sev-faf* and *sca-Gal4;UAS-faf,* both of which fail to substitute for the endogen-ous *faf* gene even partially, also express *faf* early in eye development but their expression is limited to cells within the facet.

The failure of *sev-faf* and *sca-Gal4; UAS-faf* to complement *faf* mutations is unlikely to be due to low levels of *faf*

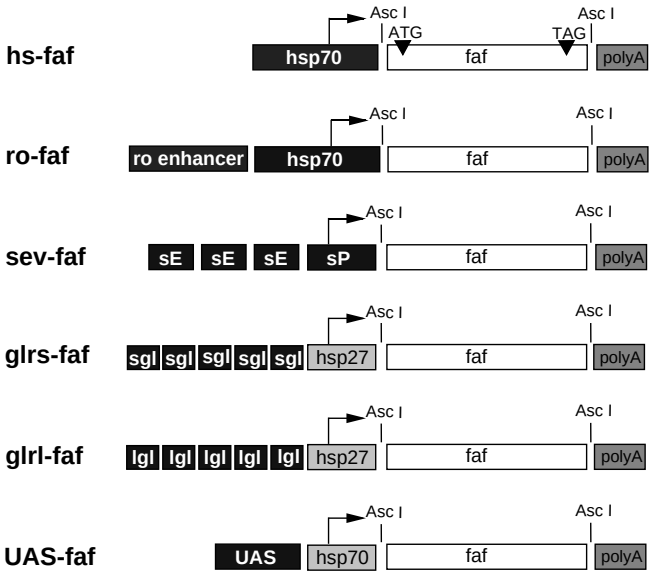
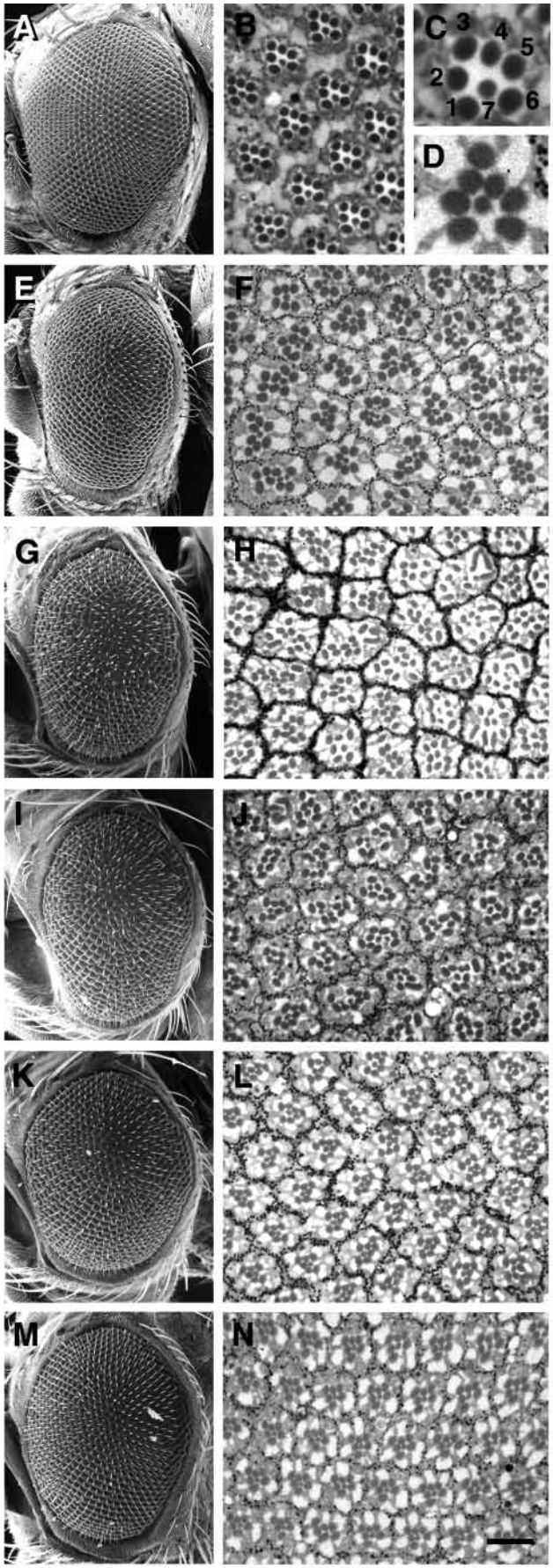


Fig. 2. Promoter-*faf* constructs. The six promoter-*faf* genes constructed are diagrammed; details of the constructions are provided in Materials and Methods. Arrows indicate the start of transcription. The positions of *Asc*I sites flanking the *faf* cDNA are indicated. ATG and TAG indicate the start and stop codons, respectively, within the *faf* cDNA. Abbreviations: hs, heat shock; ro, *rough*; sev, *sevenless*; glrs, *glass*-responsive short; glrl, *glass*-responsive long; UAS, upstream activation sequence; hsp70, *heat-shock protein 70* gene promoter in *ro-faf* and the TATA box and transcription start site only in *UAS-faf*; polyA, polyadenylation sequences from SV40; sE, *sev* enhancer; sP, *sev* promoter; sgl, short *glass*-binding site; hsp27, *heat-shock protein 27* gene TATA box region and transcription start site; lgl, long *glass*-binding site.



expression. The eye discs of flies transformed with promoter-*lacZ* hybrid genes, in which the promoters are essentially identical to those driving *faf* expression, were histochemically stained for β-galactosidase activity (Materials and Methods). These experiments revealed that the *sca-Gal4; UAS* and the *glrs* promoter constructs are the strongest, and that the *ro* and *sev* promoter constructs are of similar strength (Materials and Methods).

In addition, the promoters used are regulated normally in *faf* mutant larval eye discs, except for the inevitable differences due to the developmental defects in *faf* mutant discs (Fig. 4).

The precise timing of *faf* expression is not critical

The endogenous *faf* gene is expressed ahead of and posterior to the furrow, but not within the furrow (Fischer-Vize et al., 1992a). Although *ro-faf* is expressed in a nearly complementary pattern, it can substitute functionally for the endogenous *faf* gene (Table 1; Fig. 3). Thus, the precise timing of *faf* expression is not crucial to its function.

The essential functions of *faf* are not temporally separable

There are several aspects to the *faf* mutant facet phenotype (Fischer-Vize et al., 1992a; Huang et al., 1995); most facets contain one or more extra outer photoreceptor cells, however, facets often also contain extra Sevenless-independent (J. A. F.-V., unpublished data) R7-like cells or are missing R7 cells. In order to determine whether the partial rescue observed with some *ro-faf* and all *glrs-faf* transformant lines was due to rescue of only one or two of the three mutant facet defects, the retinas of partially rescued *ro-faf; faf^{BX4}* and *glrs-faf; faf^{BX4}* eyes were examined. In all cases, partially rescued eyes contained facets with each of the three types of defects (Fig. 3L and data not shown). Thus, *faf* does not have critical functions separable temporally or spatially in these experiments; early expression of *faf* is sufficient to circumvent the three kinds of defects in *faf* mutant eyes.

A short pulse of *faf* expression is nearly sufficient for normal eye development

The experiments described above suggest that *faf* activity might have a lasting effect because expression of *faf* in cells within the furrow *(ro-faf)* is manifested later by the exclusion of the mystery cells from the precluster. Flies containing *hs-faf* genes in a *faf^{BX4}* mutant background were used to investigate how early in eye disc development cells could respond to Faf activity.

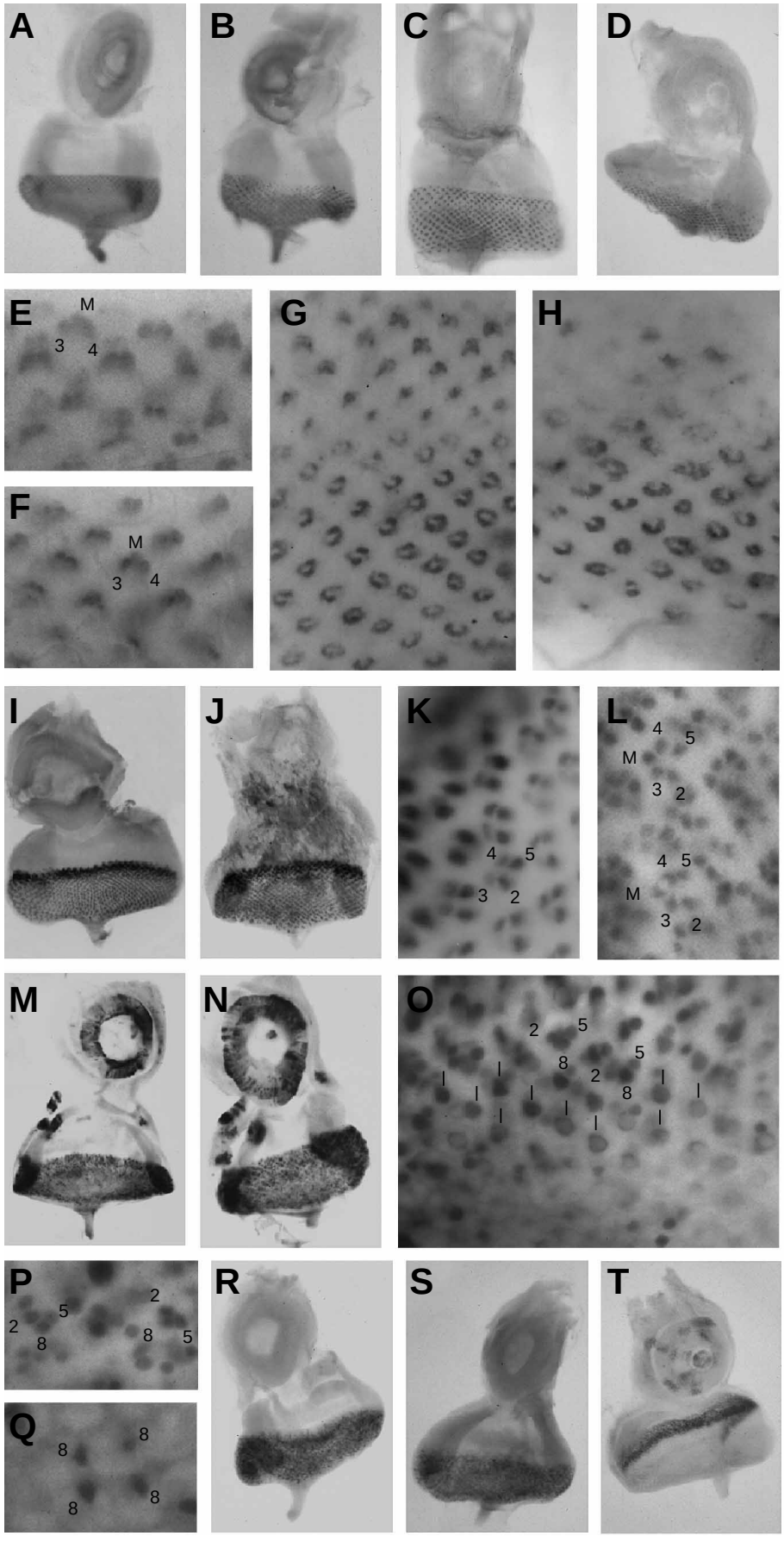
Developing *hs-faf; faf^{BX4}* embryos, larvae and pupae were given a single heat shock at 24 hour intervals after egg laying

Fig. 3. Complementation of *faf^{BX4}* by promoter-*faf* genes. Scanning electron micrographs of the external eyes (A,E,G,I,K,M) and tangential sections (B,C,D,F,H,J,L,N) of eyes representative of the genotypes indicated below are shown. (A-C) wild-type; (D-F) *faf^{BX4}*; (G,H) *sca-Gal4/ UAS-faf; faf^{BX4}*; (I,J) *sev-faf; faf^{BX4}*; (K,L) *glrs-faf; faf^{BX4}*; (M,N) *ro-faf; faf^{BX4}*. Anterior is to the left in all of the scanning electron micrographs. C and D are enlargements of individual facets in B and F, respectively. Numbers in C indicate photoreceptor cells R1-R7. The bar in N represents ~20 μm in B, F, H, J, L and M, ~10 μm in C and D and ~140 μm in A, E, G, I, K and M.

(AEL), allowed to develop into adults and their external eyes examined (for details, see Materials and Methods). The morphogenetic furrow begins to move during the early third instar. Flies heat shocked as embryos or first instar larvae were indistinguishable from non-heat-shocked *hs-faf; faf*^{BX4} or *faf*^{BX4} flies (Fig. 5A and data not shown). In contrast, flies heat shocked as third instar larvae (72-96 hours AEL) often had nearly completely wild-type eyes, with just a little roughness (~6 rows) at the anterior of the eye, the final facets to complete assembly (Fig. 5C). Later heat shocks resulted in eyes with some roughness at the posterior (Fig. 5B). We interpret these latter eyes as having expressed *faf* after the furrow had already moved and thus they contain a few rows of facets where the facet preclusters developed without benefit of *faf* activity.

How can one pulse of *faf* expression be sufficient for a nearly completely normal eye? These results could be explained in either of two ways: either Faf protein is extremely stable or some or all cells in a third instar larval eye disc are competent to respond stably to Faf activity. The latter interpretation is strongly favored because there are three arguments against Faf protein stability. First, the Faf protein sequence contains several putative PEST sequences (Fischer-Vize et al., 1992a), which are characteristic of short-lived proteins (Rogers et al., 1986). Second, a Faf/LacZ fusion protein containing the first 402 amino acids of Faf protein is unstable as it disappears abruptly in the morphogenetic furrow (Fischer-Vize et al., 1992a). Finally, the results of the heat-shock experiments argue against Faf protein being extremely stable. In order for Faf protein stability to account for rescue of nearly the entire eye with one heat shock, Faf protein would have to be stable for 56 hours (see Materials and Methods). If so, then flies heat shocked at 48 hours AEL should have had ~10 rows of wild-type facets (see Materials and Methods) and rescue was never observed in these flies.

In order to investigate the stability of Faf protein, protein extracts from *hs-faf* transformant embryos harvested at different times after heat shock were assayed for Faf protein. The Faf protein expressed from the *hs-faf* gene is tagged with the ‘Myc epitope’ (Materials and



Methods) and thus Faf protein could be detected using antibodies to the Myc epitope in Western blotting experiments. As shown in Fig. 6, large amounts of Faf protein are detected 4 hours after heat shock, the level of Faf decreases dramatically by 8 hours and no Faf protein is detected by 12 hours after heat shock.

Thus, Faf stability cannot account for the ability of one heat shock to rescue nearly the whole *faf* mutant eye to wild-type. Rather, it appears that undifferentiated cells in the third instar larval eye disc are competent to respond to Faf activity and their response is lasting. Either all cells anterior to the 7-cell precluster stage are competent to respond to Faf, or some cells respond and initiate a self-perpetuating process involving continual communication with more anterior cells as the furrow progresses.

Fig. 4. Expression patterns of *sev, ro, glrs, sca* and *h* in *faf* mutant eye discs. Shown are third instar larval eye-antennal discs stained with a variety of antibodies to show the patterns of expression of either promoter-*lacZ* reporter genes or endogenous genes in wild-type and *faf* mutant backgrounds. (Expression of the promoter-*faf* constructs could not be assayed directly because anti-Faf antibodies useful for this assay do not exist and the Myc-epitope tag present in the Faf protein is also not useful for whole-mount staining of eye discs.) (A-H) The *sev* expression pattern: (A) *sev-lacZ* and (B) *sev-lacZ; faf*^{FO8} discs stained with anti-β-galactosidase (β-gal) antibodies, (C) wild-type and (D) *faf*^{FO8} discs stained with anti-Sev antibodies. In *faf* mutant discs, the early *sev* expression pattern is easier to visualize using *sev-lacZ* and anti-β-gal antibodies, while the late pattern is easier to visualize in both wild-type and *faf* mutant discs using anti-Sev antibodies (Tomlinson et al., 1987; Bowtell et al., 1989). All staining in A-D is behind the morphogenetic furrow. (E,F) Enlargements of portions of A and B, respectively, near the morphogenetic furrow, showing β-gal expression in the mystery cells (M) and photoreceptors R3 and R4. (G,H) Enlargements of portions of C and D, respectively, showing Sev protein expression near the furrow (top half of panel) through the posterior of the disc (bottom half of panel). Early expression of Sev in the mystery cells, R3 and R4 is patchy in *faf* mutant discs (top of H), probably because Sev protein is localized apically and the photoreceptor cells in *faf* mutant discs are shaped abnormally. In contrast, the β-gal protein in *sev-lacZ* discs is cytoplasmic and it is clear from F that *sev-lacZ* is expressed in the mystery cells, R3 and R4 in *faf* mutant discs. Sev expression in the bottom half of G and H is predominantly in R7 and the cone cells. As the identities of the individual cells are normally determined by their positions, it is not possible to identify the individual cells expressing Sev in *faf* mutant discs; the facet orientations are abnormal in the posterior of *faf* discs and there are often too few or too many cone cells (K. Mosley and J. A. F.-V., unpublished observations). However, it is clear from H that there is abundant expression of Sev in the posterior of *faf* mutant discs. (I-L) The *ro* expression pattern: (I) wild-type and (J) *faf*^{FO8} discs stained with anti-Ro antibodies. The line of strongest staining is within the morphogenetic furrow. (K,L) Enlargements of I and J, respectively, showing Ro expression in the nuclei of R2, R5, R3 and R4 and also in the mystery cells (M) in *faf* mutant discs (J). (M-Q) The *sca* expression pattern: (M) *sca-Gal4/ UAS-nuclacZ* and (N) *sca-Gal4/ UAS-nuclacZ; faf*^{FO8}*/faf*^{BX4} discs stained with anti-β-galactosidase antibodies. In both wild-type and *faf* mutant discs, staining first appears in groups of cells starting at the posterior edge of the morphogenetic furrow. This pattern is several rows delayed compared with endogenous Sca expression, which starts just anterior to the furrow (Baker et al., 1990; Mlodzik et al., 1990). (O) An enlargement of a portion of M near the morphogenetic furrow is shown. β-gal expression is detected predominantly in the nuclei of R8 (some R8 nuclei are indicated by lines), also in R2 and R5, and sometimes in other R-cells (not shown). (P,Q) Enlargments of portions of N near the furrow (P) and in the middle of the disc (Q) showing β-gal expression in the nuclei of R8, R2 and R5. Unlike the R8 nuclei in wild-type discs, the R8 nuclei are not in uniform apical/basal positions in *faf* discs, so that only a few R8 nuclei are in the same plane of focus in one field. Enlargements of the expression pattern within the facet preclusters at the posterior edge of the furrow are not shown due to the difficulty in getting structures within the furrow in one focal plane. (R,S) *glrs-lacZ* (R) and *glrs-lacZ; faf*^{FO8} (S) discs stained with anti-β-gal are shown. Staining is present in all cells starting at the posterior edge of the morphogenetic furrow. (T) A *faf*^{FO8} disc stained with anti-H antibodies is shown. As in wild-type discs (not shown; Brown et al., 1991), a band of cells anterior to the furrow is stained.

Ectopic *faf* expression is inconsequential

The *ro-faf*, *glrs-faf* and *hs-faf* constructs were designed to test for an effect of ectopic *faf* expression in the morphogenetic furrow. Ectopic expression has no detrimental effect on eye development as otherwise wild-type flies transformed with any of the three constructs show no observable eye defects (data not shown). In fact, furrow expression alone can substitute nearly completely for the normal pattern of *faf* expression (see above).

The *faf* gene is normally expressed in some tissues other than the eye, but its expression is not ubiquitous (Fischer-Vize et al., 1992a). The *hs-faf* gene and *UAS-faf* gene in combination with *Gal4* insertions in the *sca*, *elav* and *h* genes all express *faf* ectopically outside the eye with no obvious effects in a wild-type background.

Interactions between *faf* and other genes

In order to determine whether *faf* regulates any known pathway affecting photoreceptor determination in the developing eye, mutations in genes involved in cell cycle regulation and the Notch, Ras and DER signaling pathways, in addition to other genes, were tested for enhancement and suppression of the *faf* mutant eye phenotype (see Materials and Methods). Loss-of-function mutations in many genes were tested for their ability to dominantly enhance the weak rough eye phenotype of *faf*^{FO8}*/faf*^{BX3} flies; *faf*^{FO8} is a very strong mutant allele and *faf*^{BX3} is a very weak allele (Fischer-Vize et al., 1992a). The *faf*^{FO8}*/faf*^{BX3} background is known to be highly sensitive to 50% reduction in the levels of other as yet unidentified gene products, as this background was used successfully in a genetic screen for enhancers of *faf* (J.A.F.-V. and S. Leavell, unpublished data). Suppression was assayed in a *faf*^{FO8} homozygous background, which has been shown to be sensitive to suppression by reducing the level of a proteasome component by half (Huang et al., 1995). None of the mutations tested showed significant enhancement or suppression of the *faf* mutant eye phenotype.

DISCUSSION

It has been demonstrated that the deubiquitinating enzyme encoded by the *faf* gene is required in a signaling pathway emanating from outside the facet that inhibits inappropriate neural determination of the mystery cells and other cells. The precise timing of *faf* expression is not critical and ectopic *faf* expression is not detrimental to facet assembly. Finally, one pulse of *faf* expression is sufficient for normal eye development, suggesting that some or all cells in early third instar

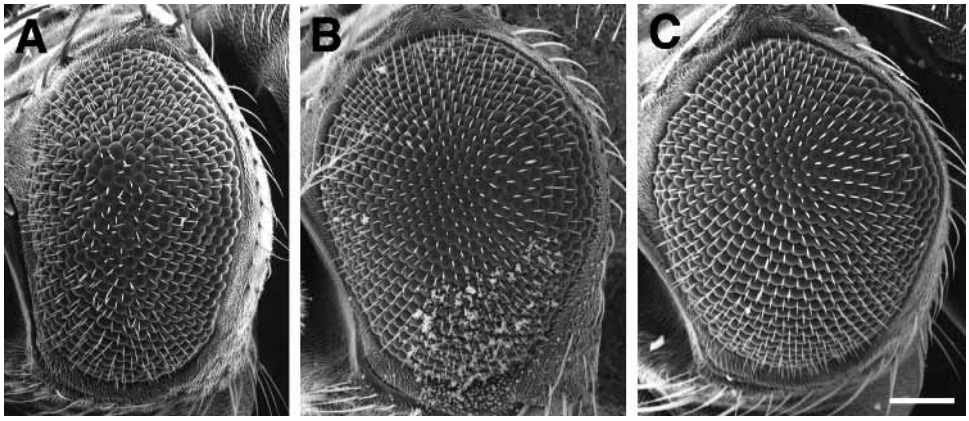


Fig. 5. Complementation of faf^{BX4} by *hs-faf*. Scanning electron micrographs of the external eyes of *hs-faf; faf*^{BX4} flies are shown. Anterior is to the left in all panels. Flies contained two copies of the *hs-faf* gene. (A) No heat shock applied. (B) Flies were heat shocked at 96-120 hours AEL. Note that the eye surface is rough at the very posterior. (C) Flies were heat shocked at 72-96 hours AEL. Note that the eye surface is rough at the very anterior. The bar in C represents ~100 μm in all panels.

larval eye discs are competent to respond to *faf* activity and that the response is lasting.

In which cells does Faf function?

The finding that *faf* works in cells outside the facet preclusters is unusual; the only other gene known to work in these cells is *groucho* (Fischer-Vize et al, 1992b; see below). The experiments presented here reveal in which cells *faf* is not absolutely required and offer strong support for one interpretation of a previous analysis of facets mosaic for *faf*+ and *faf*– photoreceptors: that is, *faf* is required in cells other than those within the 7-cell preclusters that emerge from the morphogenetic furrow. The failure of *faf* expressed in the *sev* or *sca* patterns to substitute even partially for the endogenous *faf* gene demonstrates that *faf* function is not critical in the mystery cells nor within the other precluster cells that the mystery cells contact (R8 and R3/4).

The experiments presented here do not, however, identify precisely the cells in which *faf* is essential. Analysis of mosaic facets suggests that to inhibit their misdetermination as photoreceptors, *faf* works in cells near to the mystery cells (Fischer-Vize et al., 1992a). These cells could be either in the undifferentiated cell pool surrounding the preclusters or cells within the 'rosette' structures that emerge from the morphogenetic furrow. One attractive model is that the 'core cells' within the rosette (shaded in Fig. 1) send inhibitory signals to the mystery cells, preventing them from determination as neurons. However, the action of Faf may precede the appearance of the core cells behind the furrow, as *ro-faf* expressed in the furrow is sufficient for normal eye development.

In what cell communication pathway does Faf function?

There are several known genes with mutant phenotypes similar to *faf* in the retina: *argos* (Freeman et al., 1992; Kretschmar et al., 1992; Okano et al., 1992), *yan* (Lai and Rubin, 1992; Tei et al., 1992), *Gap1* (Gaul et al., 1992; Rogge et al., 1992), *Notch* (Cagan and Ready, 1989), *groucho* (Fischer-Vize et al., 1992b), *Delta* (Park et al., 1995) and *strawberry-notch* (Coyle-Thompson and Banerjee, 1993). However, the *faf* gene does not appear to be essential in a common pathway with any of these genes.

The *faf* gene cannot be critical to the neural inhibition pathway involving Argos because Argos protein is secreted by photoreceptor cells within the facet as a consequence of DER activation (Schweitzer et al., 1995a,b; Golembo et al., 1996); a *sev-argos* gene can substitute for endogenous *argos* (Freeman, 1995) while *sev-faf* is completely ineffective as a replacement for the endogenous *faf* gene. Similarly, Faf cannot be essential for the regulation of any gene product that functions within the precluster, such as Yan and Gap1. Unlike Faf, both Yan and Gap1 function cell autonomously to repress the development of the mystery and cone cells as R7s (Lai and Rubin, 1992; Gaul et al., 1992; Rogge et al., 1992); *sev-faf* expresses *faf* in the mystery cells and cone cells with no effect on the ectopic R7 phenotype of *faf* mutants. In addition, no significant genetic interactions were detected between *faf* and *yan* or *Gap1* mutations.

The *Notch*, *Delta*, *groucho* and *sno* genes are all involved in the *Notch* signaling pathway and *groucho* is known to function in cells outside the facet. The failure to detect genetic interactions between *faf* mutations and mutations in *Notch* pathway genes suggests that *faf* is not likely to be a critical regulator of the *Notch* pathway.

One plausible hypothesis is that *faf* facilitates the function of a short-range inhibitor of DER, similar to Argos, that emanates from the core cells or other cells outside the 7-cell precluster (see Fig. 1). However, the failure to detect strong genetic interactions between *faf* and DER pathway genes

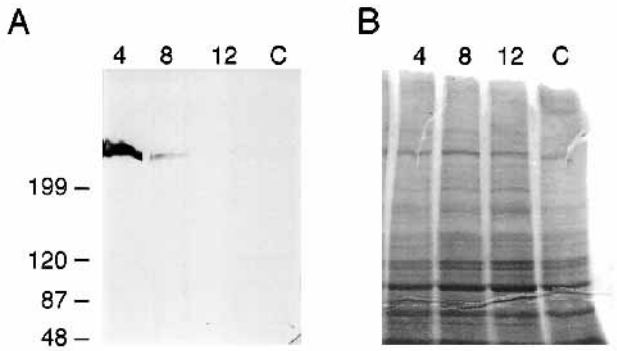


Fig. 6. Faf protein stability. (A) Immunoblot of proteins present 4, 8 and 12 hours after heat shock in *hs-faf* transformant embryos. A protein corresponding to the approximate size of Faf (~300×10^3 M_r) is detected with anti-Myc epitope antibodies at 4 and 8 hours after heat shock, but not after 12 hours, nor in control (C) embryos that were not heat shocked. Each lane contains 150 μg of total protein. (B) A Coomassie-stained protein gel of the same extracts used in A showing that none of the extracts is degraded. Each lane contains 40 μg of total protein.

suggests that *faf* may regulate an unknown inhibitory pathway for neural determination in the eye.

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