The \textit{fat facets} gene is required for \textit{Drosophila} eye and embryo development

JANICE A. FISCHER-VIZE\textsuperscript{1,2,3,}, GERALD M. RUBIN\textsuperscript{1} and RUTH LEHMANN\textsuperscript{3}

\textsuperscript{1}Howard Hughes Medical Institute, Department of Molecular and Cellular Biology, University of California at Berkeley, Berkeley, California 94720, USA
\textsuperscript{2}Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138, USA
\textsuperscript{3}Whitehead Institute for Biomedical Research, Howard Hughes Medical Institute and Department of Biology, Massachusetts Institute of Technology, 9 Cambridge Center, Cambridge, Massachusetts 02142, USA

*Author for correspondence at present address: Department of Zoology, University of Texas at Austin, Austin, Texas 78712, USA

Summary

In a screen for mutations affecting \textit{Drosophila} eye development, we have identified a gene called \textit{fat facets} (\textit{faf}) which is required for cell interactions that prevent particular cells in the developing eye from becoming photoreceptors. Analysis of eyes mosaic for \textit{faf}\textsuperscript{+} and \textit{faf}\textsuperscript{−} cells shows that \textit{faf} is required in cells near to, but outside, normal developing photoreceptors and also outside of the ectopic photoreceptors in mutant facets. \textit{faf} is also essential during oogenesis, and we show that a \textit{faf}-lacZ hybrid protein is localized via the first 392 amino acids of \textit{faf} to the posterior pole of oocytes. Posterior localization of \textit{faf}-lacZ depends on \textit{oskar}. \textit{oskar} encodes a key organizer of the pole plasm, a specialized cytoplasm at the posterior pole of embryos. The pole plasm is required for germ cell formation and contains the determinant of posterior polarity, encoded by \textit{nanos}. Although other pole plasm components are required for localization of \textit{nanos} RNA or for \textit{nanos} protein function, \textit{faf} is not. We have cloned the \textit{faf} gene, and have shown that it encodes two similar large (~300×10\textsuperscript{3} M\textsubscript{r}) proteins that are unique with respect to other known proteins.

Key words: \textit{fat facets}, \textit{Drosophila}, eye development, oogenesis, neurogenesis, protein localization.

Introduction

Short-range cell interactions guide the assembly of the \textit{Drosophila} compound eye in the eye imaginal disc, as cells sequentially join growing clusters of unit eyes, or facets (recently reviewed by Banerjee and Zipursky, 1990; Rubin, 1991). Many genes have been identified that are involved in particular steps of the assembly pathway, for example, \textit{scabrous}, \textit{retina aberrant in pattern}, \textit{rough}, \textit{Star}, \textit{Bar}, \textit{seven-up}, \textit{sevenless}, \textit{seven in absentia}, \textit{bride-of-sevenless}, and \textit{glass} (Baker et al., 1990; Mlodzik et al., 1990a; Karpi- low et al., 1989; Tomlinson et al., 1988; Saint et al., 1988; Heberlein and Rubin, 1991; Higashijima et al., 1992; Mlodzik et al., 1990b; Hafen et al., 1987; Carthew and Rubin, 1990; Reinke and Zipursky, 1988; Moses et al., 1989). The products of all of these genes are required in specific cells within the growing facets, where they either participate in cell-cell interactions or control cell determination from within the cell. The large number of such genes supports the model that facet assembly is controlled by cells within the facets (Tomlinson and Ready, 1987a).

In recent studies of the \textit{argos} (Freeman et al., 1992) and \textit{groucho} (\textit{gro}; Fischer-Vize et al., 1992) genes, it was shown that cells outside of the developing facets also influence facet assembly. Mutations in both genes have a similar eye phenotype; a few cells are inappropriately determined as photoreceptors. \textit{argos} encodes a diffusible factor (Freeman et al., 1992) and \textit{gro} encodes a nuclear protein (Delidakis et al., 1991). Nothing more is certain about the mechanism of either gene product’s function in the eye, or their relationship to each other.

Here we describe a gene called \textit{fat facets} (\textit{faf}), that also influences facet assembly from outside the facet. \textit{faf} mutant eyes contain one or more ectopic photoreceptor cells in nearly every facet. By analyzing facets mosaic for \textit{faf}\textsuperscript{+} and \textit{faf}\textsuperscript{−} photoreceptors, we show that \textit{faf} is required in cells near to, but outside the eight photoreceptors in wild-type facets, and outside the extra photoreceptors in mutant facets. Unlike \textit{argos}, \textit{faf} does not appear to be diffusible. Unlike the pleiotropic roles of \textit{gro}, the essential role of \textit{faf} in repression of neural cell determination is limited to the eye disc.

\textit{faf} is also required during oogenesis. Embryos from \textit{faf} mutant mothers never cellularize, except for the formation of a few primordial germ cells, called pole cells, at the posterior of the embryo. Moreover, we find that a \textit{faf}-lacZ hybrid protein is localized to the posterior pole of oocytes.

The localization of particular mRNAs and proteins to
opposite poles of the oocyte is the mechanism by which anterior-posterior polarity of the embryo is established (reviewed by St. Johnston and Nüsslein-Volhard, 1992). Posterior polarity depends on ten posterior group genes, nine of which have been organized into a functional hierarchy: cappuccino (capu) and spare (spire), staufen (stau), oskar (osk), vasaf (vas), tudor (tud), valois (vls), nanos (nos) and pumilio (pum) (Boswell et al., 1991; Manseau and Schüpbach, 1989; St. Johnston et al., 1991; Ephrussi et al., 1991; Kim-Ha et al., 1991; Ephrussi and Lehmann, 1992; Hay et al., 1990; Lasko and Ashburner, 1990; Lehmann and Nüsslein-Volhard, 1991, 1987; Macdonald, 1992; D. Barker, J. Moore, L.K. Dickinson and R.L., unpublished data). The posterior group genes function through the nos gene product, the posterior determinant, by localizing nos RNA to the posterior of the embryo, or modulating the function of nos protein. Except for nos and pum, the posterior group genes are also required for pole cell formation (reviewed by Lehmann, 1992). Stau and vas proteins, and osk RNA and protein are known to be localized to the posterior of oocytes prior to nos RNA, and the localization of each depends on the genes upstream in the hierarchy (St. Johnston et al., 1991; Ephrussi et al., 1991; A. Ephrussi and R.L., unpublished data; Hay et al., 1988b; Lasko and Ashburner, 1990). Nothing is known about the mechanism of protein localization to the posterior of the oocyte.

Here we show that a faf-lacZ hybrid protein containing only the first 392 amino acids of faf is posteriorly localized as a protein, and that its localization depends on osk. Unlike the posterior group genes, faf is not required for nos RNA localization or function. We have cloned the faf gene, and have shown that it encodes two large (~300x10^3 M_r) proteins with different carboxy termini, unique with respect to other known proteins.

Materials and methods

Drosophila genetics

Fly lines

The enhancer trap line AE127, marked with white*, has a P element in the svp gene (M. Mlodzik, J. Heilig and G.M.R., unpublished data). The posterior group mutant alleles used were capu^Rk and spire^AP (Manseau and Schüpbach, 1989), stau^D3, osk^560, osk^46 vas^D1, va$^D11$ (Lehmann and Nüsslein-Volhard, 1991), stau^HL54, tuf^cwc8, vls^R871 (Schüpbach and Wieschaus, 1986) and pum^690 (Lehmann and Nüsslein-Volhard, 1987). All other mutant markers are described in Lindels and Zimm (1992). Flies were grown on standard food at 25°C.

Generation of faf alleles

Six EMS-induced faf alleles were isolated by crossing mutagenized bw; st males to bw; st faf^O9/TM6B virgins, and screening approximately 27,000 bw; *st/st faf^O9 male progeny for rough eyes. Thirteen additional faf alleles were induced with X-rays as follows. st males were exposed to X-rays (4000 rads) and then crossed to bw; marb^CD4 st cu sr e faf^O9/TM6B virgins. (marb^CD4 is another rough eye mutation; J. A. F.-V. and G. M. R., unpublished data). Approximately 30,000 bw; *st/*...faf^O9 male progeny were screened for rough eyes. One additional faf allele (faf^El1) was isolated in a P-M hybrid dysgenesis screen. Birm2; st males were crossed to bw; st faf^O9/TM6B virgins at 16-18°C. (The Birm2 and A(2-3)99B chromosomes are described by Robertson et al., 1988). The male progeny (Birm2*/4; A(2-3)/st) were crossed to bw; marb^D4 st cu sr e faf^O9/TM6B virgins at 22°C, and >100,000 st/*...faf^O9 male progeny were screened for rough eyes.

Meiotic mapping of faf

First, bw; st faf^O9/Th st cu sr e ca virgins were crossed with bw; th st cu sr e ca males. Many progeny of all genotypic classes were individually mated with bw; st faf^O9/TM6B virgins to determine whether or not the recombinant chromosome contained faf^O9. By this process, faf^O9 was positioned near the tip of chromosome 3R. Next, faf^O9 was found to be distal to a white* P element (P[w^+]) insertion in polytene chromosome region 99A (a gift of the Rubin laboratory enhancer trappers) by crossing w; st e faf^O9/P[w^+] virgins to w; st e faf^O9 males and scoring many progeny for the e, w* and faf* phenotypes.

Other fly crosses

Standard genetic crosses were used to combine faf^O9 with AE127, the posterior group genes with a faf/lacZ P element on the X chromosome, and faf, fafII and fafIII P elements on chromosomes X and II with faf^BX4.

Generation of faf* eye clones

Clones of faf^O9/faf^O9 cells marked by the absence of the white gene product (no pigment granules associated with photoreceptor or pigment cells) were generated by crossing w^118; st faf^O9/TM6B males with w^118; P[w^+]/D; D3; 1018; P[w^+]/D; 3R. Next, faf^O9/TM6B virgins and X-irradiating (1000 rads) their progeny as first instar larvae. P[w^+]/D3; 1018; P[w^+]/D3; 1018; faf^O9/TM6B produced many progeny for which the faf alleles were isolated. The walk was monitored by using the most distal recombinant phage isolated from each step as a probe for in situ hybridization to faf^O9/TM6B chromosomes. After a phage hybridized to both ends of the faf^O9 inversion, we walked another two steps and then attempted to locate, within the walk, DNA lesions associated with each of the homozygous viable faf alleles. Blots of genomic DNA prepared from flies homozygous for each faf allele and restricted with either EcoRI or BamHI were sequentially hybridized with recombinant phage spanning the entire 83 kb walk, and lesions associated with faf^B13 and faf^B12 were identified.

Isolation of faf genomic and cDNA

Unless otherwise noted, all molecular biology was carried out using standard procedures (Sambrook et al., 1989). The starting point for an 83 kb chromosomal walk (see Pirrotta, 1986) through the faf locus was a 5.2 kb Drosophila genomic DNA fragment adjacent to a P element in polytene region 100D (a gift of the Rubin laboratory enhancer trappers) isolated by 'genomic rescue' (Pirrotta, 1986). We walked within a non-amplified Sau3A-partial Drosophila genomic DNA library in bacteriophage Agem12 (Promega), constructed from genomic DNA of the isogenic st strain in which the faf alleles were originally isolated. The walk was monitored by using the most distal recombinant phage isolated from each step as a probe for in situ hybridization to faf^O9/TM6B chromosomes. After a phage hybridized to both ends of the faf^O9 inversion, we walked another two steps and then attempted to locate, within the walk, DNA lesions associated with each of the homozygous viable faf alleles. Blots of genomic DNA prepared from flies homozygous for each faf allele and restricted with either EcoRI or BamHI were sequentially hybridized with recombinant phage spanning the entire 83 kb walk, and lesions associated with faf^B13 and faf^B12 were identified.

Seven different partial faf cDNAs were isolated from an eye disc cDNA library (constructed by Alan Cowman) using a 4.5 kb EcoRI fragment of genomic DNA as a hybridization probe. All of the cDNAs were subcloned into the Bluescript plasmid (Stratagene) as EcoRI fragments. The relationships between the seven cDNAs were determined by hybridizing DNA blots of cDNA restriction fragments with probes prepared from each cDNA. The cDNAs were mapped onto the genomic DNA by using cDNAs as
hybridization probes to blots of restricted recombinant phage clones spanning the walk.

**DNA sequencing and analysis**

The DNA sequence of cDNAs 3-2 (~3.1 kb) and 7-3 (~5.8 kb), subcloned into the Bluescript plasmid (Strategen), were determined on one strand from a plasmid primer by constructing nested sets of deletions with exonuclease III and S1 nuclease (Erase-A-Base Kit, Promega) and using Sequenase (USB). The sequence of the 3′-most 0.5 kb of cDNA 6-5 was determined starting with a plasmid primer and continually synthesizing 17-mer oligonucleotide primers corresponding to the end of each sequence. The opposite strand of the genomic DNA sequence was determined using 17-mer oligonucleotide primers synthesized based on the cDNA sequence. Additional primers were synthesized to sequence through introns. DNA sequences were compiled and analyzed using the MacVector programs. Database searches for nucleic acid and protein sequence similarities were performed at the NCBI BLAST network service (PIR, SwissProt, Genpept, and GPUpdate databases).

**Plasmid constructions**

DNA manipulations were carried out using standard procedures (Sambrook et al., 1989). The three genomic DNA plasmids (rafI, rafII, rafIII) were constructed by piecing together subcloned EcoRI and BamHI fragments of various recombinant λ phage clones, and then cloning them as NotI fragments into the P element transformation vector pW8 (Klenzenger et al., 1987) in the same orientation as the white gene. To construct rafI/lacZ, raf coding sequences ending at the BglII site after the first 392 amino acids were joined to a BamHI site at the start of the lacZ coding region. A 4.2 kb XbaI-BglII fragment of rafIII (the 5′-most 4.2 kb with an engineered XbaI site at the EcoRI site) was ligated with a 4.0 kb BamHI-EcoRI fragment of pC4βgal (Thummel et al., 1988; containing lacZ coding sequences and poly(A) addition sequences from SV40), into a plasmid vector restricted with XbaI and EcoRI. The resulting 8.2 kb fragment and cloned into pW8. raf/lacZ was generated from rafI/lacZ by cloning sequences encoding a nuclear localization signal (KKKRKV; Kalderon et al., 1984) in frame into the XhoI site as an adaptor for another BglII and annealed 44-mer oligonucleotides with one XbaI and one BamHI end (5′-CTAGAAACATGACCCCCCCCAAGGAGCCGCAAGTTGGAGGAC and 5′-GATCGTCCTCCACCTTGCCGCTTCTTGGGGGTTGGTATGGTT) were ligated into the adaptor. In total, 19 amino acids were added.

**RNA blot analysis**

Total nucleic acid was prepared from 5 ovaries or 20 eye disc complexes by dissecting the tissues in PBS and immediately transferring them to a microfuge tube containing 200 μl of homogenization buffer (50 mM Tris pH 7.5, 50 mM NaCl, 10 mM EDTA, 0.5% SDS, 2 mg/ml proteinase K). The tissues were homogenized with a small teflon homogenizer, incubated at 37°C for 45 minutes and extracted with phenol/chloroform, which was then back-extracted with homogenization buffer. The pooled supernatants were ethanol precipitated, the pellets washed with 100% ethanol and resuspended in formamide gel loading buffer (Sambrook et al., 1989). The total nucleic acid preparations were electrophoresed on formaldehyde gels, blotted and hybridized with 32P-labeled, double-stranded DNA probes according to standard procedures (Sambrook et al., 1989).

**P element transformation**

w1118 embryos were injected according to standard procedures (Spradling, 1986) with each of the fas genomic DNA plasmids and fas-lacZ hybrid plasmids at 300 μg/ml, and the helper plasmid pCE7A2-3 at 100 μg/ml. Plasmids for injections were purified on CsCl-EtBr equilibrium density gradients (Sambrook et al., 1989). Transgenic flies were identified by their red eye color, mapped to a chromosome and balanced using standard genetic crosses.

**Sections and scanning electron microscopy of adult eyes**

Eyes were fixed, embedded in plastic and sectioned essentially as described in Tomlinson and Ready, 1987b. Heads were bisected to ensure penetration of fix. Sections were 1 μm. Scanning electron microscopy was performed as described in Kimmel et al., 1990.

**β-galactosidase histochemistry**

The procedures followed for β-galactosidase histochemical staining are essentially those of Y. Hiromi and M. Mlodzik (personal communication). Ovaries and eye discs were dissected in PBS, fixed in a depression slide for 15 minutes at room temperature in 1% glutaraldehyde in PBS, and then washed with PBS at least twice for 10 minutes. The wash was replaced by prewarmed (65°C for at least 15 minutes) staining solution (10 mM NaPO4 (pH 7.2), 150 mM NaCl, 1 mM MgCl2, 3 mM K3[Fe(CN)6], 3 mM K4[Fe(CN)6]) and then by prewarmed staining solution to which 1/30 volume of 8% X-Gal in DMSO was added. Ovaries were incubated for 5 hours or less and eye discs overnight at room temperature. The staining reaction was stopped by several rinses in PBS and the tissues were then transferred to 80% glycerol in PBS, and mounted in this solution. Ovaries and eye discs from wild-type (non-transgenic) flies were used as negative controls. Embryos were dechorionated with 50% bleach, washed with H2O and fixed in a depression slide for 15 minutes in 0.5 ml heptane saturated with 25% glutaraldehyde in PBS. Embryos were transferred to a glass slide and excess heptane blotted. After the heptane evaporated, embryos were transferred to double-sided tape on another glass slide by gently sandwiching the embryos between two slides. Embryos were then covered with PBS, devitellinized with a tungsten needle and transferred to a depression slide. After removing the PBS, embryos were incubated at room temperature in staining solution (above) without X-Gal for 5 minutes, and then in staining solution with X-Gal. fas/lacZ embryos began to stain almost immediately. Stained embryos were quickly rinsed in 70% and then 100% ethanol and mounted in 80% glycerol in PBS.

**Immunostaining eye discs**

Third instar larval eye disc complexes, attached only to the mouth hooks, were dissected in PBS. All antibody incubations and washes between them were performed in 96-well microtiter dishes, and eye discs were transferred from well to well with tungsten hooks (A. Tomlinson, personal communication). For mAb22C10, discs were treated exactly as described in Tomlinson and Ready, 1987a. mAbro staining was performed by a modification of the procedure in Kimmel et al., 1990. Discs were fixed for 45-55 minutes on ice (0.1 M Pipes (pH 7.0), 2.0 mM EGTA, 1.0 mM MgSO4, 4% paraformaldehyde), washed for at least 15 minutes on ice in PBS+0.1% Triton X-100 (PBST), and then incubated in mAbro diluted in PBST for at least 1 hour at 4°C. After three 5
minute washes in PBST, discs were transferred to biotin-conjugated goat anti-mouse secondary antibody (Jackson) diluted in PBST and incubated for at least 1 hour at 4°C. Following three 5 minute washes in PBST, discs were transferred to ABC complex (Vectastain Elite Kit, Vector) diluted in PBST for exactly 1 hour, washed three times for 5 minutes in PBST and put on ice.

Discs were incubated in HRP-staining solution (1× PBS, 0.1% Triton X-100, 0.5 mg/ml DAB, 0.02% CoCl₂, 0.02% NiCl₂, 0.003% H₂O₂) for ~1 minute while monitoring the level of staining with a dissecting microscope, and then transferred to 1× PBS. Discs were transferred to 30% ethanol, the peripodial membranes were removed and then the discs were dehydrated (50, 70, 90, 2× 100% ethanol) and mounted in DPX (Fluka).

The probes were a 2.1 kb fragment of cyclinB cDNA and linear Bluescript plasmid (Stratagene) as a negative control. Embryos and ovaries were dehydrated and mounted using exactly the same procedure as for the immunostained embryos.

### Results

#### Identification of faf alleles

The two original faf alleles (faf^FO8 and faf^BB12) were identified as mutations causing rough eyes in an extensive screen of the *Drosophila* third chromosome for recessive viable mutations with abnormal eye morphology (J.A.F.-V., R.W. Carthew and G.M.R., unpublished data; see Fischer-Vize et al., 1992). Subsequently, 20 additional alleles, generated chemically, with X-rays, or by hybrid dysgenesis-induced mutations were examined for visible lesions (Materials and methods). All of the strong faf alleles also result in female sterility. The eyes of flies expressing faf^BX3 and faf^BX4 appear weakly rough. The eyes of flies carrying each of the other weak alleles vary from weakly rough to wild-type. Only three of the six weak faf alleles that result in a weakly rough eye also cause female sterility. This implies that there are different requirements for faf function, either for levels of faf activity or for specific functional domains of faf protein, in the eye and ovary. No intermediate maternal effect phenotypes were observed; the phenotypes of embryos from each of the alleles also vary from weakly rough to wild-type.

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Eye phenotype</th>
<th>Female sterility</th>
<th>Cytology</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS-induced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>faf^FO8</td>
<td>strong</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td>faf^BB12</td>
<td>strong</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td>faf^B8</td>
<td>strong</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td>faf^B4</td>
<td>weak</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td>faf^B5</td>
<td>weak</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td>faf^B6</td>
<td>weak</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td>faf^B7</td>
<td>weak</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td>faf^B8</td>
<td>weak</td>
<td>sterile</td>
<td></td>
</tr>
<tr>
<td>X-ray-induced</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>faf^BX1</td>
<td>strong</td>
<td>sterile</td>
<td>no defect</td>
</tr>
<tr>
<td>faf^BX2</td>
<td>weak</td>
<td>sterile</td>
<td>no defect</td>
</tr>
<tr>
<td>faf^BX3</td>
<td>weak</td>
<td>sterile</td>
<td>no defect</td>
</tr>
<tr>
<td>faf^BX4</td>
<td>strong</td>
<td>sterile</td>
<td>In(3R)100E/98D</td>
</tr>
<tr>
<td>faf^BX5</td>
<td>lethal</td>
<td>n.a.</td>
<td>no defect</td>
</tr>
<tr>
<td>faf^BX6</td>
<td>lethal</td>
<td>n.a.</td>
<td>no defect</td>
</tr>
<tr>
<td>faf^BX7</td>
<td>weak</td>
<td>fertile</td>
<td>N.D.</td>
</tr>
<tr>
<td>faf^BX8</td>
<td>strong</td>
<td>sterile</td>
<td>no defect</td>
</tr>
<tr>
<td>faf^BX9</td>
<td>lethal</td>
<td>n.a.</td>
<td>*</td>
</tr>
<tr>
<td>faf^BX10</td>
<td>lethal</td>
<td>n.a.</td>
<td>no defect</td>
</tr>
<tr>
<td>faf^BX11</td>
<td>weak</td>
<td>fertile</td>
<td>no defect</td>
</tr>
<tr>
<td>faf^BX12</td>
<td>lethal</td>
<td>n.a.</td>
<td>*</td>
</tr>
<tr>
<td>faf^BX13</td>
<td>lethal</td>
<td>n.a.</td>
<td>N.D.</td>
</tr>
<tr>
<td>faf^BX14</td>
<td>lethal</td>
<td>n.a.</td>
<td>N.D.</td>
</tr>
<tr>
<td>faf^BX15</td>
<td>strong</td>
<td>sterile</td>
<td>N.D.</td>
</tr>
<tr>
<td>hybrid dysgenesis-induced faf^Bp</td>
<td>lethal</td>
<td>n.a.</td>
<td>Df(3R)100EF</td>
</tr>
</tbody>
</table>

Twenty-two independent alleles of faf were generated using EMS, X-rays, or P-M hybrid dysgenesis (Materials and methods). Except for the two original alleles, faf^FO8 and faf^BB12, all of the faf alleles were identified as flies with rough eyes carrying a mutagenized chromosome in *trans* to faf^FO8 (Materials and methods). All of the strong faf alleles also result in female sterility. The eyes of flies expressing faf^BX3 and faf^BX4 appear weakly rough. The eyes of flies carrying each of the other weak alleles vary from weakly rough to wild-type. Only three of the six weak faf alleles that result in a weakly rough eye also cause female sterility. This implies that there are different requirements for faf function, either for levels of faf activity or for specific functional domains of faf protein, in the eye and ovary. No intermediate maternal effect phenotypes were observed; the phenotypes of embryos from each of the alleles also vary from weakly rough to wild-type.

#### Immunostaining embryos

Embryos were dechorionated in 50% bleach, washed in H₂O and fixed by gentle shaking for 20 minutes in 1 part fix (0.1 M Pipes pH 7.0, 2 mM MgSO₄, 1 mM EGTA, 4% formaldehyde) : 3 parts heptane. The fix was removed and embryos devitellinized by adding an equal volume of methanol and shaking hard. Devitellinized embryos were rinsed twice with methanol, then washed three times for 30 minutes with BBT (1× PBS, 0.1% BSA, 0.1% Tween20) and incubated at 4°C overnight with preadsorbed primary antibody (anti-vas polyclonal rabbit sera or anti-αβ polyclonal rat sera) diluted in BBT. Embryos were then washed four times for 30 minutes with BNT (PBS + 0.2% goat serum) and incubated for 2 hours in preadsorbed biotin-conjugated goat anti-rabbit (for anti-vas) or biotin-conjugated goat anti-rat (for anti-αβ) secondary antibody (Jackson) diluted in BBT. After at least three 30 minute washes in PBT (PBS + 0.1% Tween 20) embryos were incubated for exactly 1 hour in ABC complex (Vectastain Elite Kit, Vector), washed three times in PBT, then put on ice. Embryos were incubated for a few minutes in HRP staining solution (0.5 mg/ml DAB +0.006% H₂O₂ in PBT) and the reaction stopped by washing with PBT. Embryos were dehydrated with ethanol (30, 50, 70, 90, 95, 2× 100%), rinsed for 30 seconds in acetone and mounted in Araldite (Ladd).
genesis (Table 1), were identified as rough-eyed flies carrying the mutagenized chromosome in trans to faf<sup>kos</sup> (Materials and methods). Based on the severity of eye roughness of homozygotes, the faf alleles were categorized as strong or weak (Table 1 and see below).

Meiotic mapping positioned faf very close to the tip of chromosome 3R (Materials and methods). faf was specifically localized to polytene region 100E by analyzing the polytene chromosomes of several faf alleles (Table 1) and finding that faf<sup>BX4</sup> is an inversion with breakpoints in polytene regions 98D and 100E, and faf<sup>BP</sup> is deficient for the entire 100EF region (data not shown).

The faf mutant phenotype

Eye phenotype

The eyes of faf mutant flies appear rough due to irregularities in the normally precise hexagonal array of facets (Fig. 1A,B). In order to investigate the faf mutant phenotype beneath the eye surface, the retinas of faf flies were examined in tangential sections. The wild-type retina is a hexagonal lattice of identical facets, or ommatidia (Fig. 1C). An ommatidium has eight photoreceptor cells (R-cells), each uniquely positioned within a trapezoidal array. There are six outer photoreceptor cells, R1-R6, with large light-gathering organelles, called rhabdomeres, and two inner R-cells, R7 and R8, with small rhabdomeres. The trapezoids are oriented in one direction and are symmetrical with respect to an equator. Many defects are present in strong faf mutant retinas, the most striking of which is the appearance of one, two or occasionally three extra outer photoreceptor cells in most facets (Fig. 1D and legend).

The retinas of all strong faf mutants appear similar (data not shown). The retinas of all weak faf alleles are also similar to each other and generally have as few as 1% mutant facets (data not shown; Table 1 legend). The eye defects of the strong alleles are likely to represent the complete loss-of-function (null) eye phenotype of faf, as the strong mutants (including the viable inversion faf<sup>BX4</sup> that breaks within the faf transcript (see below)) show the same phenotype when homozygous or in trans to the faf<sup>BP</sup> deficiency (data not shown).

Maternal effect phenotype

All of the strong faf alleles and three of the weak alleles cause female sterility (Table 1 and legend). Females homozygous for any of these alleles have apparently normal ovaries, but lay eggs that never form cuticle and never hatch. In order to examine the mutant embryos in more detail, embryos of wild-type and homozygous faf<sup>kos</sup> mothers were stained consecutively with the DNA stain Hoechst to reveal nuclei, and with rhodamine-conjugated phalloidin, which stains f-actin and thus allows visualization of cell membranes (Materials and methods). No normal embryos from faf<sup>kos</sup> mothers were ever observed. After the fertilized egg is laid, the nucleus normally undergoes 14 synchronous cycles of division prior to cellularization (see Foe and Alberts, 1983). By division cycle 10, the nuclei have migrated to the egg periphery to form the ‘syncytial blastoderm’ (Fig. 2A) and the primordial germ cells, called pole cells, located at the posterior pole of the embryo, form (Fig. 2B). The rest of the nuclei continue synchronous divisions until cycle 14, when cellularization produces the ‘cellular blastoderm’. In wild-type embryos, f-actin is associated with each nucleus at syncytial blastoderm (Fig. 2B), and then with cell membranes at cellular blastoderm (Fig. 2F: Karr and Alberts, 1986). In embryos from faf mutant mothers (hereafter called faf mutant embryos), no normal syncytial blastoderm embryos are observed. The embryo shown in Fig. 2.C,D appears to be at least in cycle 10 because pole cells are present (Fig. 2D), but most of the nuclei have not migrated to the periphery (Fig. 2C). Also commonly observed were embryos in which patches of asynchronously dividing nuclei had migrated to the periphery (Fig. 2G). Except for the pole cells (which are fewer in number and
spread out instead of grouped together as in wild-type - see Fig. 11D, below), no cellularization of \textit{faf} mutant embryos was ever observed (Fig. 2H).

In summary, the most obvious defects in \textit{faf} null mutants are in the eye and in the embryo. In the eye, the most striking abnormality is the appearance of ectopic outer photoreceptors. \textit{faf} mutant embryos never reach normal syncytial blastoderm and except for some pole cells, never cellularize.

**Developmental defects in \textit{faf} mutant larval eye discs**

To determine if defects in early stages of ommatidial assembly contribute to the \textit{faf} eye phenotype, wild-type and strong \textit{faf} mutant larval eye discs were stained with the neural specific antibody mAb22C10 (Fujita et al., 1982). mAb22C10 reveals the sequence of photoreceptor cell assembly (R8, R2/5, R3/4, R1/6, R7) as each R-cell expresses the mAb22C10 antigen when it acquires neural identity (Fig.3A; Tomlinson and Ready, 1987a). As ommatidial development proceeds in a posterior-to-anterior wave in the eye disc, facets at all stages of photoreceptor cell assembly are observed in one larval disc. The most striking defect observed in stained \textit{faf} mutant larval eye discs is the appearance of one or two ectopic photoreceptor cells in most developing facets at the time when R3/4 normally begin to stain with mAb22C10 (Fig.3B,C). These cells may be the mystery cells, which are normally positioned between R3 and R4 in a precluster that emerges from the morphogenetic furrow, but then disappear into the surrounding pool of undifferentiated cells without expressing neural antigens (Tomlinson and Ready, 1987a; Wolff and Ready, 1991a).

In order to determine if the ectopic photoreceptors are indeed positioned between R3 and R4, we examined the expression of the \textit{rough} (\textit{ro}; Tomlinson et al., 1988; Saint et al., 1988) and \textit{seven-up} (\textit{svp}; Mlodzik et al., 1990b) genes in \textit{faf} larval eye discs. Both \textit{ro} and \textit{svp} are normally expressed in R3 and R4 (Kimmel et al., 1990; Mlodzik et al., 1990b; Fig.3 legend). In \textit{faf} mutant eye discs, both genes are also expressed in one or two cells adjacent to R3 and R4. Thus, the ectopic R-cells in \textit{faf} mutant larval eyes are likely to be the mystery cells.

**\textit{faf}^+ function is required outside normal and ectopic photoreceptors**

In order to determine which cells require \textit{faf}^+ function to exclude the ectopic R-cells from the precluster, marked (\textit{w^c}) clones of \textit{faf} \textit{FO8} mutant cells in wild-type eyes were generated using X-ray induced somatic recombination.

---

**Fig. 3.** The mystery cells become ectopic photoreceptors in \textit{faf} mutant eye discs. Wild-type (A) and \textit{faf} \textit{FO8} (B,C) larval eye discs were stained with mAb22C10 (Materials and methods) to reveal the sequence of photoreceptor cell assembly. mAb22C10 stains photoreceptor cell cytoplasm and the discs are photographed to show the apical tips of the photoreceptor cells near the morphogenetic furrow. The arrows point along the direction of the morphogenetic furrow. Five-cell clusters containing R8,2,5,3,4 are apparent in wild-type (A) discs. At a similar distance from the furrow in \textit{faf} mutant discs (B), clusters containing 6 or 7 cells are seen. The cell(s) which appear to be the extra photoreceptors (see below), situated between R3 and R4, are indicated by asterisks (*). Improperly spaced clusters (C) also appear occasionally in \textit{faf} mutant discs. (D) \textit{faf} mutant larval eye discs stained with an antibody to the ro protein (Materials and methods), which is expressed in the nuclei of R2,5,3,4. One or two ectopic nuclei (*), apparently located between R3 and R4, express ro. (E) \textit{faf} mutant larval eye discs carrying one copy of the enhancer trap P element \textit{AE127}, which is inserted in the \textit{svp} gene and expresses β-galactosidase in the nuclei of R3,4,1,6, were stained with an antibody to β-galactosidase (Materials and methods). A cluster is shown containing an ectopic nucleus (*), apparently between R3 and R4. As the mystery cells are normally located between the R3/4 pair, the ectopic photoreceptor cells are likely to be improperly determined mystery cells. \textit{ro} and \textit{svp} appear to be otherwise appropriately expressed, suggesting that many of the photoreceptor cells in \textit{faf} mutant discs acquire their normal identities. The bar (in C) is 10 µm in all panels.
Fig. 4. *faf* 

(A) A tangential section through a typical *w*-*f Ruf* clone in a *w*-*f Ruf* eye, generated by X-ray-induced somatic recombination (Materials and methods). The *w* clone is recognizable by the absence of pigment granules in the pigment cells forming the hexagonal lattice, and also near each photoreceptor (black dots). The large arrow points to the *w* ectopic cell in a phenotypically mutant mosaic facet at the clone border. The small arrow indicates a phenotypically wild-type facet at the clone border. (B) A tabulation of data obtained by examining both phenotypically wild-type and phenotypically mutant mosaic facets in many different clones. The R-cell genotypes in 209 wild-type mosaic facets in 20 separate clones were scored. (For technical reasons, a smaller number of R8 cells was scored.) As there are normally ~10% wild-type facets in an *f Ruf* mutant eye, if there were no requirement for *f Ruf* in particular R-cells, those R-cells would be expected to be *w*-*f Ruf* at a frequency of ~55% [50%+(1/2)(10%)]. In contrast, if *f Ruf* were required in particular R-cells in order to form a wild-type facet, those R-cells would be expected to be *f Ruf* at a frequency of ~95% [100%- (1/2)(10%)=95%]. The data indicate that all R-cells are *f Ruf* at a similar frequency, averaging at 65%. This slightly higher than random (55%) frequency suggests that *f Ruf* is required in cells closely related by lineage to (near to) the R-cells (see Tomlinson et al., 1988; Reinke and Zipursky, 1988; Carthew and Rubin, 1990; Mlodzik et al., 1990b.) Phenotypically mutant facets were also examined, and the tabulation of the frequency of *w*-*f Ruf* outer R-cells in 78 mosaic facets at the borders of 18 different clones is shown. In contrast to the results obtained with the wild-type mosaic facets, the frequency of *f Ruf* outer R-cells in the mutant mosaic facets (35%) is lower than random [50%- (1/2)(10%)=45%], suggesting that either R-cells within these facets or cells related by lineage to them must be *f Ruf* in order to produce a phenotypically mutant facet. (C-I) Phenotypically mutant mosaic facets in which one or two extra photoreceptors are neatly added. In these facets, knowing that the extra cells are the mystery cells, located between R3 and R4, it is possible to identify all of the outer R-cells. We assume that no gross rearrangement of the R-cells occurs in the eye disc after the assembly stages that we have observed. The ectopic cell (labeled ‘m’ for mystery cell) and also sometimes either R3, R4 or both, are *w*-*f Ruf*. The facets in E and F also contain *w* R8’s (not visible in this plane of section). The facet in H has two ectopic R-cells, only one of which is *w*. These facets show that *f Ruf* expression in the ectopic cells or in the cells they contact (R8,3,4) is not sufficient to exclude the mystery cells from the facet. We observed a total of five facets like those shown in C and D, and six additional facets in which the ectopic cell was *w*, and four facets where R8 could be scored definitively as *w*. No phenotypically mutant facets were observed in which all R-cells were *w*. We interpret this as a reflection of the proximity of the *f Ruf*-requiring cells to the mystery cells, and thus the low probability of the cell(s) requiring *f Ruf* function being *w* while the mystery cells and all of the other R-cells in a mutant facet are *w*. 

### Table: Phenotypically Wild-type Mosaic Facets

<table>
<thead>
<tr>
<th>Number</th>
<th>% w-f Ruf</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>129/205</td>
</tr>
<tr>
<td>R2</td>
<td>131/205</td>
</tr>
<tr>
<td>R3</td>
<td>127/205</td>
</tr>
<tr>
<td>R4</td>
<td>136/205</td>
</tr>
<tr>
<td>R5</td>
<td>132/205</td>
</tr>
<tr>
<td>R6</td>
<td>142/205</td>
</tr>
<tr>
<td>R7</td>
<td>141/205</td>
</tr>
<tr>
<td>R8</td>
<td>63-69</td>
</tr>
</tbody>
</table>

### Table: Phenotypically Mutant Mosaic Facets

<table>
<thead>
<tr>
<th>Number</th>
<th>% w-f Ruf</th>
</tr>
</thead>
<tbody>
<tr>
<td>228/550</td>
<td>35</td>
</tr>
</tbody>
</table>
Within the patch of \( w^{+} \text{faf}^{-} \) cells, the retina shows the \( \text{faf} \) mutant phenotype, and outside the \( w^{-} \) clone, the retina appears wild type (Fig. 4A). As there are no lineage restrictions on cells within particular facets (Ready et al., 1976; Lawrence and Green, 1979; Wolff and Ready, 1991b), there are mosaic ommatidia at the clone borders, containing both \( w^{+} \text{faf}^{+} \) and \( w^{-} \text{faf}^{-} \) photoreceptor cells (Fig. 4A). Some of the mosaic facets are phenotypically wild type (that is, there are two inner and six outer R-cells) and some are typical \( \text{faf} \) mutant facets, with more than six outer R-cells (Fig. 4A).

Examination of both the phenotypically wild-type and mutant mosaic facets shows that \( \text{faf}^{+} \) function is not required in photoreceptor cells. First, the genotypes of the different photoreceptors in phenotypically wild-type mosaic ommatidia were scored to determine if there is a tendency for particular photoreceptors to be \( \text{faf}^{+} \). None of the eight photoreceptors was \( \text{faf}^{+} \) nearly as frequently as expected if \( \text{faf}^{+} \) function is required in a particular photoreceptor cell (Fig. 4B and legend). Second, we find that in the phenotype of the mosaic, the ectopic photoreceptor cells are not always \( \text{faf}^{-} \), and the R-cells that they contact (R3, R4 and R8) are also not always \( \text{faf}^{-} \), consistent with the analysis of the wild-type mosaics (above). Thus, it is not the absence of \( \text{faf}^{+} \) function in the ectopic cells, or the cells they contact, that results in their misdetermination as photoreceptors.

The analysis of phenotypically wild-type and mutant facets suggests that \( \text{faf} \) is required in cells closely related by lineage to (that is, near to) the photoreceptors. Each of the photoreceptors in the phenotypically wild-type mosaic ommatidia are \( \text{faf}^{+} \) at a higher than random frequency (Fig. 4B and legend). In contrast, the outer photoreceptors within the mutant mosaic ommatidia are \( \text{faf}^{+} \) at a lower than random frequency (Fig. 4B and legend).

In summary, we interpret these observations as indicating that cells near to, but outside the normal or ectopic photoreceptors in a particular facet must be \( \text{faf}^{+} \) in order to prevent the neuralization of the mystery cells.

Cloning the \( \text{faf} \) gene

Having localized \( \text{faf} \) to polytene region 100E (above), we isolated \( \text{Drosophila} \) genomic DNA adjacent to a P element inserted in 100D (Materials and methods) and used this DNA as a starting point for an 84 kb chromosomal walk within a bacteriophage lambda \( \text{Drosophila} \) genomic DNA library, towards the telomere of 3R (Materials and methods, Fig. 5A). Progress in the walk was monitored by hybridizing, in situ, phage clone probes from each step to polytene chromosomes of the homozygous viable inversion \( \text{faf}^{++} \) (Table 1, Materials and methods). A phage clone hybridized to both ends of the inversion about 55 kb into the walk (Fig. 5A; data not shown). An additional 29 kb of genomic DNA was isolated distal to the inversion breakpoint. The \( \text{faf}^{++} \) breakpoint was localized to a 1.8 kb \( \text{EcoRI-BamHI} \) fragment of genomic DNA and a small deletion in a neighboring 1.5 kb \( \text{BamHI} \) fragment was found to be associated with \( \text{faf}^{++} \) (Fig. 5A; data not shown - see Materials and methods). A 4.5 kb \( \text{EcoRI} \) fragment of genomic DNA containing the region of both mutant lesions was used to isolate many cDNAs from an eye imaginal disc cDNA library (Materials and methods). All of the cDNAs are either identical to or contained within the three shown in Fig. 5A (Materials and methods). DNA sequencing (below) of the three cDNAs as indicated in Fig. 5A revealed that the cDNAs represent two transcripts of at least ~8500 and ~8900 nt, which differ in their 3′ ends. (We do not know whether the 3′ end of the cDNA 3-2 represents the actual start of the \( \text{faf} \) transcript.) This result correlates with an analysis of RNA blots (Fig. 5B) hybridized with the 4.5 kb \( \text{EcoRI} \) fragment, which shows two transcripts of slightly different size in the appropriate range. Conceptual translation of the two transcripts reveals long open reading frames differing at their 3′ termini (see below).

In order to show more conclusively that the cDNAs correspond to the \( \text{faf} \) gene, transgenic \( \text{Drosophila} \) lines were generated by P element transformation with the three different fragments of genomic DNA shown in Fig. 5A (Materials and methods). One copy of any of the three genomic DNA fragments complements completely both the eye and female sterile mutant phenotypes of \( \text{faf}^{BX4} \) (data not shown; see Materials and methods). In addition, buds of eye and eye disc RNA probed with all regions of the smallest complementing genomic DNA fragment (\( \text{faf}^{III} \)) reveal only the two transcripts that hybridize to the 4.5 kb \( \text{EcoRI} \) fragment of genomic DNA (Fig. 5A). Thus, these are the only two RNAs transcribed within \( \text{faf}^{III} \) that are present in eye discs or ovaries.

Predicted \( \text{faf} \) protein sequences

The DNA sequences of three overlapping partial cDNAs were determined (Fig. 5A and Materials and methods). In order to confirm the cDNA sequences, the corresponding genomic DNA sequence was determined on the opposite strand A composite sequence is shown in Fig. 6. The genomic DNA sequence shows that the \( \text{faf} \) gene is composed of at least 17 exons. Conceptual translation of the two classes of cDNAs reveals proteins of 2711 and 2747 amino acids, differing slightly in their carboxy termini due to an alternate splice near the 3′ end of the primary transcript. Apart from their large size, the \( \text{faf} \) proteins have few remarkable features. At amino acids 262-290, there is a potential leucine zipper structural domain, shown to be a dimerization site within transcription factors (Landschulz et al., 1988). As the leucine repeat is not adjacent to a particularly basic (DNA-binding) domain, \( \text{faf} \) is unlikely to be a transcription factor. In addition, there is a likely PEST sequence (Rogers et al., 1986), often found in rapidly degraded proteins, at amino acids 18-34. No other significant similarity with another protein or DNA sequence in multiple databases has been found (Materials and methods).

Expression of \( \text{faf-lacZ} \) hybrid proteins in the eye disc

In order to determine which cells in the eye and eye disc express \( \text{faf} \), we ‘tagged’ the \( \text{faf} \) protein with the \( \text{E. coli} \) gene encoding β-galactosidase (lacZ) by constructing the two hybrid genes shown in Fig. 7A (Materials and methods). One hybrid, \( \text{faf/lacZ} \), contains the \( \text{faf} \) promoter through the first 392 amino acids of the \( \text{faf} \) protein (Fig. 7A legend), fused in frame to sequences encoding lacZ. The other hybrid gene, \( \text{fafnuclacZ} \), is identical to \( \text{faf/lacZ} \), except that an oligonucleotide containing a nuclear localization signal
was cloned in frame just after the first 54 amino acids encoded by faf (Materials and methods). Nuclear localization of an antigen greatly facilitates the identification of individual cells expressing it in the eye disc. Both constructs were introduced into the Drosophila genome by P element transformation (Materials and methods). Ten independent lines (five containing each construct) were analyzed as described below and showed similar results, except that $faf^{nuc/lacZ}$ lines showed nuclear expression.

Eye discs of third instar transgenic larvae were histochemically stained for $\beta$-galactosidase activity (Materials and methods). Staining was seen within the entire region ahead of the morphogenetic furrow including the antennal disc (Fig. 7B), but only after prolonged incubation. Staining eye discs with antibodies to $\beta$-galactosidase, which is more sensitive than histochemical staining, revealed an even lower level of $faf$-lacZ protein behind the furrow as well (Fig. 8A-C; Materials and methods). Because the antibody stain in $faf^{nuc/lacZ}$ lines is nuclear, it is possible to identify the cells that express $faf$-lacZ protein. $faf^{nuc/lacZ}$ eye discs stain with anti- $\beta$-galactosidase antibodies in all cells ahead of and behind the morphogenetic furrow, but not within the furrow (Fig. 8A-F). Therefore, $faf$-lacZ hybrid protein must be rapidly degraded in cells as they enter the furrow.

$faf$-lacZ protein was also consistently detected in other discs and larval and adult tissues (i.e. fat body, gut, larval ovary and testes, adult male sex organs; data not shown).

$faf$-lacZ protein, but not mRNA, is localized to the posterior pole of oocytes

Ovaries of transgenic females were also histochemically stained for $\beta$-galactosidase activity (Materials and methods). Although only one $faf$-lacZ line obtained could be tested, we are confident that the ~13 kb $faf^{III}$ fragment contains the entire $faf$ gene because its 3′ end is identical to that of $faf^{I}$ and its 3′ end is identical to that of $faf^{II}$. (B) An autoradiograph of an RNA blot revealing the two $faf$ transcripts. Each lane contains total RNA prepared from 20 third instar larval eye imaginal disc cDNA fragments or 5 ovaries of each of the three Drosophila lines indicated (Materials and methods). The autoradiograph shown was following hybridization with a 32P-labelled double-stranded RNA DNA probe corresponding to the 4.5 kb EcoRI fragment shown in (A). The bottom panel is a lighter exposure of the first four lanes. Identical results were obtained when the blot was stripped of old probe and hybridized with the upstream 3.5 kb EcoRI fragment or the downstream 6.1 kb BamHI fragment of DNA (see A). In order to approximate the sizes of the transcripts shown, the blot was also probed with labelled DNA corresponding to a spectrin gene (~13 kb) and a non-muscle myosin gene (~7 kb; Materials and methods). Both $faf$ transcripts are expressed in wild-type and $faf^{B12}$ eye discs and ovaries. As neither the size nor the level of $faf$ mRNA appears to be affected in $faf^{B12}$, this allele is likely to be a point mutation within the coding sequences. As expected (see A), only one truncated transcript is detected in $faf^{BX4}$ RNA.
Fig. 6. For legend see p. 996
Fig. 6. DNA and predicted protein sequences of the $\textit{faf}$ gene. A composite genomic and cDNA sequence of 11,975 nt of the $\textit{faf}$ locus is shown, including the region containing all three cDNAs shown in Fig. 5A, and 360 nt upstream. The sequenced nucleotides are numbered in plain type at the left, starting with 360 nt upstream of the beginning of cDNA 3-2, indicated by the G at position 361. cDNA 3-2 has 4 additional bases (CACG) upstream of that G that are not contained in the genomic sequence, which could either be an artifact of cDNA cloning, or could indicate an intron/exon boundary. We have not determined whether the G at nt 361 is the actual transcription start site. The predicted protein sequence is shown using the single letter amino acid code, the letters placed beneath the middle nucleotide of each codon. Stop codons are indicated with asterisks (*). The numbers in bold at the left refer to the protein sequence. The ATG at position 605 is probably the start of translation as there are no other ATGs upstream in the 3-2 cDNA sequence, there are stop codons in all three reading frames within the 3-2 cDNA sequence upstream of nt 605, and the sequences upstream of the ATG are a good match with the $\textit{Drosophila}$ translation initiation consensus sequence (Cavener, 1987). The PEST sequence similarity at amino acids 18-34 (Rogers et al., 1986) and the leucine-zipper substitution changing the amino acid from S to T.

Also, peripodial membrane nuclei were stained. The bar (in F) is 10 µm in D-F, 40 µm in C and 120 µm in A and B.

Fig. 8. $\textit{faf}$-lacZ expression in third instar larval eye-antennal discs. (A) An eye-antennal disc from a $\textit{fafnuc}$/$\textit{lacZ}$ transgenic third instar larvae stained with an anti-$\beta$-galactosidase antibody (Materials and methods). Nuclear staining is seen throughout both the antennal portion of the disc (a), and the eye portion (c), except within the morphogenetic furrow, indicated by the arrow. (B) A wild-type eye-antennal disc stained with an antibody raised against a ubiquitous nuclear antigen (gro; see Materials and methods) is shown to demonstrate that nuclear staining present in the furrow (arrow) is readily visible. (C) A close-up of the eye portion of the disc in A, showing the nuclear staining within assembling facets and the absence of stained nuclei within the furrow (arrow). (D-F) Enlargements of progressively more posterior portions of the disc shown in A and C (the direction of the furrow is straight across the top of all panels), showing that $\textit{fafnuc}$/$\textit{lacZ}$ is expressed in the nuclei of all photoreceptors (1-8) and cone cells (c). We could not specifically find stained mystery cell nuclei, probably because they are not usually apically located. However, stained basal nuclei of undetermined cells were observed (not shown). Also, peripodial membrane nuclei were stained. The bar (in F) is 10 µm in D-F, 40 µm in C and 120 µm in A and B.
Fig. 7. Expression of faf-lacZ in eye discs and ovaries assayed by β-galactosidase activity staining. (A) The two faf-lacZ hybrid genes introduced into the Drosophila genome by P element transformation are shown (Materials and methods). faf/lacZ contains the first 4.4 kb of the fafIII genomic DNA fragment (Fig. 5A), including ~2.1 kb upstream of the putative transcription start site (indicated by the arrow), the 5′-untranslated sequences and DNA encoding the first 392 amino acids of the faf protein, including the first two exons (black boxes), two introns and part of the third exon. The faf coding sequences are fused in frame after amino acid 392 to E. coli sequences encoding lacZ (stippled box), followed by termination sequences from SV40 (Materials and methods). fafnuc/lacZ is identical to faf/lacZ except that an oligonucleotide containing an 11 amino acid nucleic acid localization signal (hatched inverted triangle) was cloned in frame just after the first 34 amino acids (Materials and methods). (B) A histochemically stained (Materials and methods) third instar larval eye-antennal disc from a fafnuc/lacZ transgenic line shows β-galactosidase activity in the antennal portion (a) of the disc and in the eye portion (e), anterior to the morphogenetic furrow (mf). In C-I, posterior is to the right. (C-D) Portions of stained ovarioles from a faf/lacZ transgenic female are shown. β-galactosidase activity is detected in the germarium (g in panel C) and in the egg chambers at all subsequent stages of oogenesis. When the oocyte (o) becomes morphologically distinct from the nurse cells (n), the β-galactosidase activity is apparently restricted to the nurse cells, as the oocyte appears unstained at stage 8 (S8). (Stages are according to Mahowald and Kambysellis, 1980). Some faf/lacZ protein must be present in stage-8 oocytes, however, because in fafnuc/lacZ lines, stain is seen concentrated in stage-8 oocyte nuclei. At stage10 (D), β-galactosidase activity is detected not only in the nurse cells, but also at the oocyte posterior pole. The posterior staining persists through the final stage of oogenesis (E-stage 14). The same results were obtained in vls8071/vls871, tud800/tud80C, pum800/pum806 and nos27/nos27 mutant females (Materials and methods). (F) A stained embryo from a faf/lacZ transgenic mother. Maternal β-galactosidase activity persists at the posterior (see text) and becomes incorporated into pole cells (pc). (G,H) Stage-10 (G) and stage-11 (H) oocytes from an osk150/osk150 mutant female containing a faf/lacZ P element. β-galactosidase activity is present in the nurse cells, but there is none at the oocyte posterior pole. The same result was obtained in capu7/capu8, spir8/pir8 and stau8/stauNL54 females (Materials and methods). (I) A portion of an ovariule from a weak vas mutant female (vas61/vas61) carrying a faf/lacZ P element. PARTICULATE β-galactosidase staining is seen throughout the ovariule, and posteriorly localized stain is present in stage 10 oocytes. Similar results were obtained with vas61/vas61, a strong allele. (J) A portion of an ovariule from a vas61/vas61 female carrying a faf/lacZ P element, showing that the particulate staining is outside of the nucleus. We do not yet understand the significance of the particulate staining.

Fig. 9. faf transcripts in ovaries. faf transcripts were detected in wild-type ovaries by whole-mount in situ hybridization of a digoxigenin-labeled faf cDNA probe (Materials and methods). faf transcripts were detected in the germarium (G) and in the egg chambers in subsequent stages of oogenesis. At stage 8 (S8), the oocyte (o) is easily distinguishable from the nurse cells (n) and faf transcripts appear to be restricted to the nurse cells, through stage 10. At stage 10, the time that faf/lacZ protein first appears at the oocyte posterior, no posteriorly localized faf transcripts are detected.

Localization of faf-lacZ protein in oocytes depends on osk
We next asked whether posterior localization of faf-lacZ protein depends on the posterior group genes. Ovaries of females homozygous mutant for each of nine posterior group genes, that also carry a copy of the faflacZ P element, were stained for β-galactosidase activity (Materials and methods). β-galactosidase activity was detected at the oocyte posterior in vas and mutants downstream, but not in osk or mutants upstream in the hierarchy (Fig. 7G-I and legend). As stau protein and osk RNA and protein are posteriorly localized earlier than faf-lacZ protein, and vas protein is localized at about the same time, faf appears to fit into the posterior group hierarchy.

Insights into the specific roles of some of the posterior group genes were recently obtained using transgenic flies containing an osk gene whose RNA is mislocalized to the anterior pole of oocytes (Ephrussi and Lehmann, 1992). Ephrussi and Lehmann have shown that osk RNA at the anterior is sufficient to localize nos RNA and also to recruit all of the pole plasm components required for the formation of functional pole cells, which include vas and tud, but not capu, spir, stau or vls. Thus, the authors conclude that capu, spir and stau are required only to tether osk gene products to the posterior pole, while osk, vas and tud are essential for pole plasm formation and nos RNA localization. (The role of vls is uncertain.)

We find that faf-lacZ protein colocalizes with osk gene products at the anterior pole in embryos of transgenic mothers carrying one copy of faflacZ and one copy of anteriorly localized osk (Fig. 10). As faf-lacZ localization in oocytes is independent of genes downstream of osk in the hierarchy, and genes upstream of osk appear to be required only to tie osk RNA to the posterior pole, we conclude that faf-lacZ protein localization is likely to depend only on osk gene products.

Pole plasm components are posteriorly localized in faf mutant embryos
The observation that faf-lacZ protein localization depends on osk suggested that posterior localization of downstream gene products (vas protein and nos RNA) may depend on faf. cycB mRNA may also be considered downstream of faf as it is posteriorly localized, dependent on vas and genes upstream (Raff et al., 1990). Using nos and cycB cDNA probes for in situ hybridization, and an antibody to vas protein, we find that all of these gene products are posteriorly
nos transcripts were detected in 0-4 hour embryos from wild-type (A,C) and faf\textsuperscript{fos} (B,D) mothers by in situ hybridization of whole embryos with a digoxigenin-labeled nos cDNA probe (Materials and methods). (A,B) Posterior localization of nos transcripts appears identical in wild-type (A) and faf mutant (B) presynaptic blastoderm embryos. nos RNA is incorporated into pole cells in wild-type embryos (C) and also in faf mutant embryos (D). The pole cells in faf mutant embryos are fewer in number and more spread out than in wild-type. The same results were obtained with all faf alleles that cause female sterility (see Table 1) and with faf\textsuperscript{BX4}/faf\textsuperscript{BP}, the allele combination most likely to be null. The same results were obtained when embryos from faf\textsuperscript{fos} mutant mothers were hybridized with a cyclinB probe, or stained with anti-vas antibodies (Materials and methods).

Fig. 11. nos RNA is posteriorly localized in faf mutant embryos. nos transcripts were detected in 0-4 hour embryos from wild-type (A,C) and faf\textsuperscript{fos} (B,D) mothers by in situ hybridization of whole embryos with a digoxigenin-labeled nos cDNA probe (Materials and methods). (A,B) Posterior localization of nos transcripts appears identical in wild-type (A) and faf mutant (B) presynaptic blastoderm embryos. nos RNA is incorporated into pole cells in wild-type embryos (C) and also in faf mutant embryos (D). The pole cells in faf mutant embryos are fewer in number and more spread out than in wild-type. The same results were obtained with all faf alleles that cause female sterility (see Table 1) and with faf\textsuperscript{BX4}/faf\textsuperscript{BP}, the allele combination most likely to be null. The same results were obtained when embryos from faf\textsuperscript{fos} mutant mothers were hybridized with a cyclinB probe, or stained with anti-vas antibodies (Materials and methods).

nos function is unimpaired in faf mutant embryos

nos protein facilitates the expression of genes that direct the abdominal segmentation pattern by preventing translation of hunchback (hb) maternal mRNA in the posterior of the embryo (Tautz, 1988; Struhl, 1989; Irish et al., 1989; Hülskamp et al., 1989). Thus, hb protein normally accumulates only in the anterior half of the embryo. By staining faf mutant embryos with an antibody to hb protein, we find that maternal hb protein is distributed normally in faf mutant embryos (data not shown; Materials and methods). Thus, faf is unlikely to be required for nos function.

Discussion

The faf gene encodes two similar large proteins specifically required during two different developmental processes in Drosophila: compound eye assembly and oogenesis. In the larval eye disc, cells outside the assembling facets require faf for short-range cell interactions that prevent the mystery cells from becoming photoreceptors. Maternal faf protein appears to be required for nuclear migration and cellularization in early embryogenesis. In addition, a faf-lacZ protein containing only the first 392 amino acids of faf is posteriorly localized in oocytes and its localization depends on osk.

faf and cell interactions in the eye disc

The faf gene appears to function in one of several negative regulatory mechanisms in place in the larval eye disc which ensure that each facet contains only eight photoreceptor cells. We have shown that faf is involved in a process whereby the mystery cells are prevented from becoming photoreceptors by cells near to but outside the photoreceptors and mystery cells. argos, a diffusible protein and gro, a nuclear protein, appear to play roles in facet assembly similar to faf (Freeman et al., 1992; Delidakis et al., 1991; Fischer-Vize et al., 1992). Further experiments are required to determine if these three genes function in the same or different pathways. In contrast, analysis of svp mutants shows that photoreceptors R3 and R4 also repress neural-ization of the mystery cells (Mlodzik et al., 1990b).

Although our results suggest that faf is required in cells near the developing photoreceptors and mystery cells, we have not identified these cells. Presumably, when the mystery cells are influenced by the negative regulatory mechanism involving faf, they are in contact with the cells in which faf is required. The precise time during eye development when faf function influences mystery cell fate is unknown. The first structure to emerge from the morphogenetic furrow is a rosette in which 10-15 cells, including the R8,2,5,3,4 precursors and the mystery cells, form a ring around four or five core cells. The ring then opens and preclusters containing R8,2,5,3,4 precursors and the mystery cells are formed (Wolf and Ready, 1991a). Thus, the cells that require faf to prevent neurogenesis of the mystery cells could be the core cells or cells adjacent to the mystery cells within the ring. Alternatively, if the faf-dependent process occurs later, uncommitted epithelial cells surrounding the precluster could be involved. We also cannot rule out the possibility that faf acts anterior to the morphogenetic furrow.

The expression of ro and svp by the mystery cells in faf mutant eye discs is consistent with the hypothesis that they are R3/4 subtype photoreceptors. In gro mutant eye discs, it was shown more conclusively that the mystery cells become R3/4 cells (Fischer-Vize et al., 1992). This repeated observation is interesting because experiments with ro show that the normal R3 and R4 cells require inductive cues from R2 and R5 in order to join the facet (Tomlinson et al., 1988). The mystery cells are located between R3 and R4 and are not in contact with R2 and R5. Their determination as R3/4 subtype photoreceptors suggests either that the inductive signal from R2/5 is permissive for photoreceptor cell determination and R3/4 subtype determination is a type of default state, or that R3/4 can send the same instructive signals as R2/5 (see Fischer-Vize et al., 1992).

The pattern of faf-lacZ expression in the larval eye disc

The expression pattern of the faf-lacZ hybrid protein in the larval eye disc is unusual; faf-lacZ is expressed in all cells anterior and posterior to the morphogenetic furrow. This pattern is consistent with the conclusion that faf is required in cells outside the early developing ommatidial clusters. It is possible that faf expression in cells within the assem-bling facets could be required for faf-dependent aspects of
eye morphogenesis other than excluding the mystery cells, such as orientation and spacing of facets.

The absence of faf-lacZ protein within the morphogenetic furrow indicates that it must be rapidly degraded in cells as they enter the furrow and suggests two possibilities. First, normal faf protein in the eye disc may be unstable, and factors that stabilize it or activate its transcription may be absent within the morphogenetic furrow. Alternatively, there may be a factor present in the furrow that actively degrades faf protein. faf-lacZ protein turnover may be mediated by the PEST sequence contained within the first 392 amino acids of faf. Some caution is warranted in interpreting lacZ hybrid protein expression patterns. However, as lacZ is generally more stable than endogenous Drosophila proteins in similar experiments (for example, see Mlodzik et al., 1990a), the normal faf protein is likely to be absent within the furrow.

Functions of faf in oogenesis

The localization of faf-lacZ protein to the posterior of oocytes and its dependence on osk suggests that faf has an important function at the posterior pole. (Although there may be stability differences between faf-lacZ and normal faf protein, we assume that the normal faf protein is similarly localized.) As nas RNA is localized to and maternal \(
bh\) protein is eliminated from the posterior of \(\text{faf}\) mutant embryos, a role for \(\text{faf}\) in abdominal pattern formation, which could have been obscured by the earlier role of \(\text{faf}\) in nuclear migration or cellularization, appears unlikely. Perhaps \(\text{faf}\) plays a role in pole plasm formation. Two pole plasm components essential for pole cell formation, osk and \(\text{vas}\) proteins, are also localized to the posterior pole of oocytes (Hay et al., 1988b; Ephrussi et al., 1991; Ephrussi and Lehmann, 1992). In addition, \(\text{vas}\) protein has been shown to be part of the polar granule, a particle composed of RNA and protein, associated with pole cells (Hay et al., 1988b). Although pole cells do form in \(\text{faf}\) mutant embryos, there are fewer than the wild-type number and they are irregularly positioned. Thus, \(\text{faf}\) could play a role in pole cell determination, development or function. Although the pole cell defects observed could be a secondary consequence of the failure of nuclear migration or cellularization in \(\text{faf}\) mutant embryos, this would not preclude a specific role for \(\text{faf}\) in pole cells as well. Further experiments are required to determine if \(\text{faf}\) mutant pole cells are functional, and to determine if \(\text{faf}\) protein is part of the polar granule.

The defects in \(\text{faf}\) mutant embryos indicate that \(\text{faf}\) plays a role in nuclear migration and possibly also in cellularization. It therefore seems puzzling that faf-lacZ is detected only at the posterior pole of oocytes and embryos. The absence of localized faf protein in \(\text{faf}\) mutant embryos does not cause the global embryogenesis defects as localization depends on \(\text{osk}\) and \(\text{osk}\) mutant embryos cellularize normally. The defects in embryogenesis caused by \(\text{faf}\) mutations could be accounted for if some maternal faf protein is distributed throughout the oocyte or embryo. There is some evidence for non-localized faf-lacZ protein in early embryos stained with antibodies to \(\beta\)-galactosidase (data not shown). However, as the faf-lacZ protein may be more stable than endogenous faf, an anti-faf antibody would be useful for investigating this possibility. Interestingly, embryos from \(\text{vas}\) and \(\text{vlv}\) mothers not only lack pole cells, but sometimes also display defects in cellularization (Schüpbach and Wieschaus, 1989; Hay et al., 1990; Lehmann and Nüsslein-Volhard, 1991).

J. A. F.-V. is extremely grateful to Doug Melton (Harvard University) for his exceptional generosity in allowing me to work in his laboratory, where some of this work was carried out. We are indebted to Tod Laverty (UC Berkeley) for expert analysis of polytene chromosomes. We thank Seymour Benzer's laboratory for mAb22C10, Ulrike Heberlein for mAbro, Anne Williamson for anti-vas antibodies, Robin Wharton and Gary Struhl for anti-hb antibodies, Christos Delidakis and Spyros Artavanis-Tsakonas for anti-gro antibodies, Sima Misra for the cycillinB plasmid, Dan Kiehart's laboratory for high-molecular-weight spectrin and non-muscle myosin plasmids, the Rubin laboratory enhancer traps for fly lines AEl27 and P[w/+]100D and Anne Ephrussi for the anterior localizing osk flies. We also thank Bruce Kimmel for performing the scanning electron microscopy in Fig. 1B. Mike Simon for Fig. 1A, Bruce Kimmel and Doug Barker for their help with the computer analysis of the faf DNA sequence, and Erin O'Shea for examining the leucine-zipper motif. J. A. F.-V. is grateful to Chris Rongo, Liz Gavis, Francisco Pelegri, Doug Barker, Jen Mach and two anonymous reviewers for their comments on the manuscript, to everyone in the Lehmann lab for advice, discussion and instruction in how to look at ovaries and embryos, and to Sydney Vize for inspiring the name \(\text{faf}\) facets. J. A. F.-V. was supported by Helen Hay Whitney and Howard Hughes Medical Institute postdoctoral fellowships. Ruth Lehmann and Gerald M. Rubin are Howard Hughes Medical Institute Investigators.

References


Kim-Ha, J., Smith, J. L. and Macdonald, P. M.

Hay, B., Ackerman, L., Barbel, S., Jan, L. Y. and Jan, Y. N.

Hülskamp, M., Schröder, C., Pfeifle, C., Jäckle, H. and Tautz, D.

Hafen, E., Basler, K., Edstroem, J. E. and Rubin, G. M.

Kimmel, B. E., Heberlein, U. and Rubin, G. M.

Lawrence, P. A. and Green, S. M.

Lehmann, R. and Nüsslein-Volhard, C.


Wieschaus, E. and Nüsslein-Volhard, C. (1986). Looking at embryos. In...


(Accepted 6 September 1992)

*Note added in proof*

The GenBank Accession numbers for the sequences reported are: L04958 (short cDNA), L04959 (long cDNA), L04960 (genomic DNA).

**Fig. 2.** The phenotype of embryos from *faf* mutant mothers. Embryos, collected after 4 hours of egg-laying, from wild-type (A,B,E,F) and *faf*FO8 (C,D,G,H) mothers were labeled consecutively with the DNA stain Hoechst and rhodamine-conjugated phalloidin, which stains f-actin, and photographed using fluorescent light with either fluorescein (A,C,E,G) or rhodamine (B,D,F,H) filters to show nuclei and f-actin filaments, respectively (Materials and methods). The same embryo is shown in each of the horizontal pairs of panels. (A,B) A wild-type embryo fixed during the mitosis that concludes cycle 11. Nuclei are at the periphery (A), and as cellularization does not occur until cycle 14 (below), f-actin is seen associated with each nucleus (B; see Karr and Alberts, 1986). The posterior of the embryo at a deeper focal plane is shown to the right of B, where the pole cells (pc), which form at cycle 10, are visible. (C,D) A *faf* mutant embryo, in which few nuclei have migrated to the periphery (C) although pole cells have formed (D), indicating that cycle 10 has already been reached. (E,F) A wild-type embryo at cycle 14 (cellular blastoderm). Cellularization is apparent as f-actin outlines the cell membranes (F). (G,H) The most mature-appearing *faf* mutant embryos seen. Patches of asynchronously dividing nuclei have migrated to the periphery (G), but no cellularization is apparent (H).