Cell, Vol. 78, 137–147, July 15, 1994, Copyright © 1994 by Cell Press

The Activities of Two Ets-Related Transcription Factors Required for Drosophila Eye Development Are Modulated by the Ras/MAPK Pathway

Elizabeth M. O'Neill, Ilaria Rebay, Robert Tjian, and Gerald M. Rubin
Howard Hughes Medical Institute
Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720–3200

Summary

We show that the activities of two Ets-related transcription factors required for normal eye development in Drosophila, pointed and yan, are regulated by the Ras1/MAPK pathway. The *pointed* gene codes for two related proteins, and we show that one form is a constitutive activator of transcription, while the activity of the other form is stimulated by the Ras1/MAPK pathway. Mutation of the single consensus MAPK phosphorylation site in the second form abrogates this responsiveness. *yan* is a negative regulator of photoreceptor determination, and genetic data suggest that it acts as an antagonist of *Ras1*. We demonstrate that yan can repress transcription and that this repression activity is negatively regulated by the Ras1/MAPK signal, most likely through direct phosphorylation of yan by MAPK.

Introduction

Intercellular signaling controls many processes in multicellular organisms, including regulation of growth control, differentiation, and specification of cell fate during development. Many of these signals are received at the cell surface by transmembrane receptor proteins. Upon being bound by ligand, these receptors initiate a signal transduction cascade through the cytoplasm to the nucleus, where transcription factors that are among the ultimate targets of such signaling pathways elicit alterations in gene expression that in turn regulate cellular events. Protein tyrosine kinases are one well-characterized class of transmembrane receptors. Genetic and biochemical studies have implicated the small G protein ras and regulators of its activity as key factors in the signal transduction cascade downstream of receptor tyrosine kinases (RTKs; reviewed by Schlessinger, 1993).

The sevenless RTK is required for proper cell fate determination of the R7 photoreceptor during Drosophila eye development (reviewed by Zipursky and Rubin, 1994). Proteins implicated in the transduction of the sevenless signal include the following: the SH2 and SH3 domain–containing protein, Drk; Ras1; and regulators of Ras1 activity, such as the GTPase-activating protein GAP1 and Son of sevenless, a guanine nucleotide exchange factor. Furthermore, ectopic expression of an activated form of Ras1 in precursors to the nonneuronal cone cells is sufficient to cause these cells to adopt an R7-like fate.

A critical component downstream of Ras in many RTK signaling pathways is the mitogen-activated protein kinase or MAPK family (reviewed by Marshall, 1994). Members of this class of kinases share the feature of being activated by a MAPK kinase (MAPKK), which in turn is activated by a MAPKK kinase (MAPKKK). The protein kinase Raf functions as a MAPKKK and forms a complex with Ras, making it a likely link between Ras and this so-called MAPK cascade. Activated forms of the Drosophila MAPK, extracellular signal–regulated kinase A gene/*rolled* (*ERKA/rolled*; Brunner et al., 1994), or of *Draf*, a Drosophila Raf homolog (Dickson et al., 1992), can also give rise to supernumerary R7 cells. Among the direct targets of MAPK that have been identified in mammalian systems are several transcription factors including c-Fos (Chen et al., 1993) and NF-IL-6 (Nakajima et al., 1993). No known transcription factor targets of the MAPK cascade have been shown to act downstream of sevenless. One candidate for such a factor is the Ets-related product of the *yan* gene (Lai and Rubin, 1992; Tei et al., 1992). Genetic data suggest that *yan* acts as an antagonist of activated Ras1 (Lai and Rubin, 1992), and the yan protein contains several consensus MAPK sites.

The Ets family of transcription factors share a DNA-binding domain called the Ets domain (Wasylyk et al., 1993). In several mammalian cell culture systems, elements in Ras-responsive promoters have been identified that correspond to Ets-binding sites (EBS), suggesting that members of this family of transcription factors might act downstream of Ras (Wasylyk et al., 1991; Conrad et al., 1994). However, neither the mechanism by which Ras might activate such factors nor the identity of any specific Ets protein targets were revealed by these studies.

There is a case where an Ets-related protein has been shown to respond to an extracellular signal. In fibroblasts, there is a complex formed in response to serum stimulation that activates transcription through the serum response element (see Treisman, 1994). One of the components of this complex, Elk-1, is Ets related and becomes phosphorylated in response to serum stimulation. This phosphorylation is dependent on a 42 kDa cellular kinase that is probably MAPK, and it causes an increase in the transcriptional activation activity of Elk-1 (Marais et al., 1993; Janknecht et al., 1993).

Other Ets family members have been shown to be involved in many processes, including T cell activation (Ets-1 and Ets-2; see Wasylyk et al., 1993), and other stages of Drosophila development, including embryonic (*pointed*) and larval stages (*E74*). *pointed* is required for normal development of the Drosophila embryonic nervous system (Klämbt, 1993; Scholz et al., 1993), and adult flies transheterozygous for certain hypomorphic alleles of *pointed* have rough eyes, suggesting a role for *pointed* in eye development (Scholz et al., 1993).

Here, we examine the role of *pointed* during Drosophila eye development, analyze biochemically the transcriptional properties of both pointed and the negative regulator yan, and then investigate the relationship between the

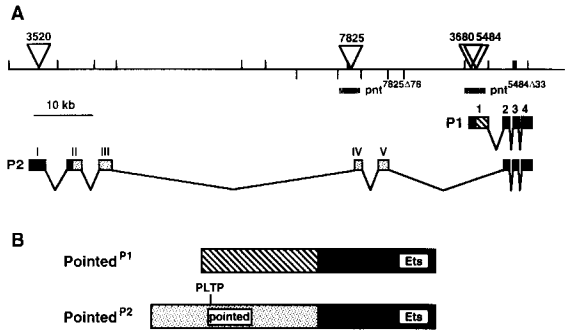


Figure 1. Genomic Organization and Structural Features of *pointed*

(A) A restriction map of the genomic region of *pointed* and a diagram of the intron/exon structure of the two transcripts derived from the *P1* and *P2* promoters are shown (Klämbt, 1993). Hatched and stippled exon structures correspond to the schematic diagrams of the protein structures in (B); closed structure indicates untranslated sequences. The precise 5′ ends of the *P1* and *P2* transcripts have not been established; the cDNAs used to construct the exon maps shown are each about a kilobase shorter than the sizes of the corresponding transcripts as estimated by RNA blots (Klämbt, 1993). Thus, either transcript might contain an additional 5′ exon. P element and exon sizes are not to scale. EcoRI sites (above the line) are according to Klämbt (1993). Only selected HindIII sites (below the line) are shown. Triangles indicate the insertion sites of P elements used in this study. The P element in line l(3)3520 is inserted 49 bp from the 3′ end of exon I. In lines l(3)3680 and l(3)5484, the P elements are approximately 1000 bp and 800 bp, respectively, upstream of the initiating ATG of the *P1* transcript. The P element in line l(3)7825 is inserted near the center of a 4.0 kb HindIII fragment that also contains exon IV. Heavy and hatched lines below the restriction map indicate approximate borders of the DNA missing in the indicated excision alleles.

(B) Schematic diagrams of the pointed^{P1} and pointed^{P2} proteins are shown. The two proteins contain a common C-terminal sequence that includes the Ets domain, but have different N-terminal sequences. Pointed^{P2} has a region of homology termed the pointed domain that is common to a subset of other Ets proteins (Klämbt, 1993). The location of the single MAPK consensus phosphorylation site (PXS/TP; Gonzalez et al., 1991; Clark-Lewis et al., 1991) in pointed^{P2} is indicated.

Ras1/MAPK pathway and these Ets-related proteins. An analysis of the eye phenotype of several classes of *pointed* alleles is presented, and genetic criteria are used to establish a role for *pointed* downstream of *Ras1* and MAPK during photoreceptor determination. To investigate the biochemical mechanism by which pointed and yan are regulated by this signaling pathway, we developed an in vitro cell culture assay that has allowed us to study the transcriptional activities of these two Ets-related proteins as well as to determine how these activities are modulated by Ras1 and MAPK.

Results

pointed Is Required for Normal Photoreceptor Determination

Many genes required for eye development are also necessary at earlier stages of development. As a result, these genes are not identified in most genetic screens for eye phenotypes. To circumvent this problem and to identify new genes required for eye development in Drosophila, we took advantage of the FLP/FRT marked mosaic system (Xu and Rubin, 1993), which allows the generation of marked clones of cells homozygous for a mutation of interest in otherwise heterozygous animals. We used this approach to screen through a collection of lethal P element enhancer trap insertions (see Experimental Procedures) for those that gave abnormal eye phenotypes in clones.

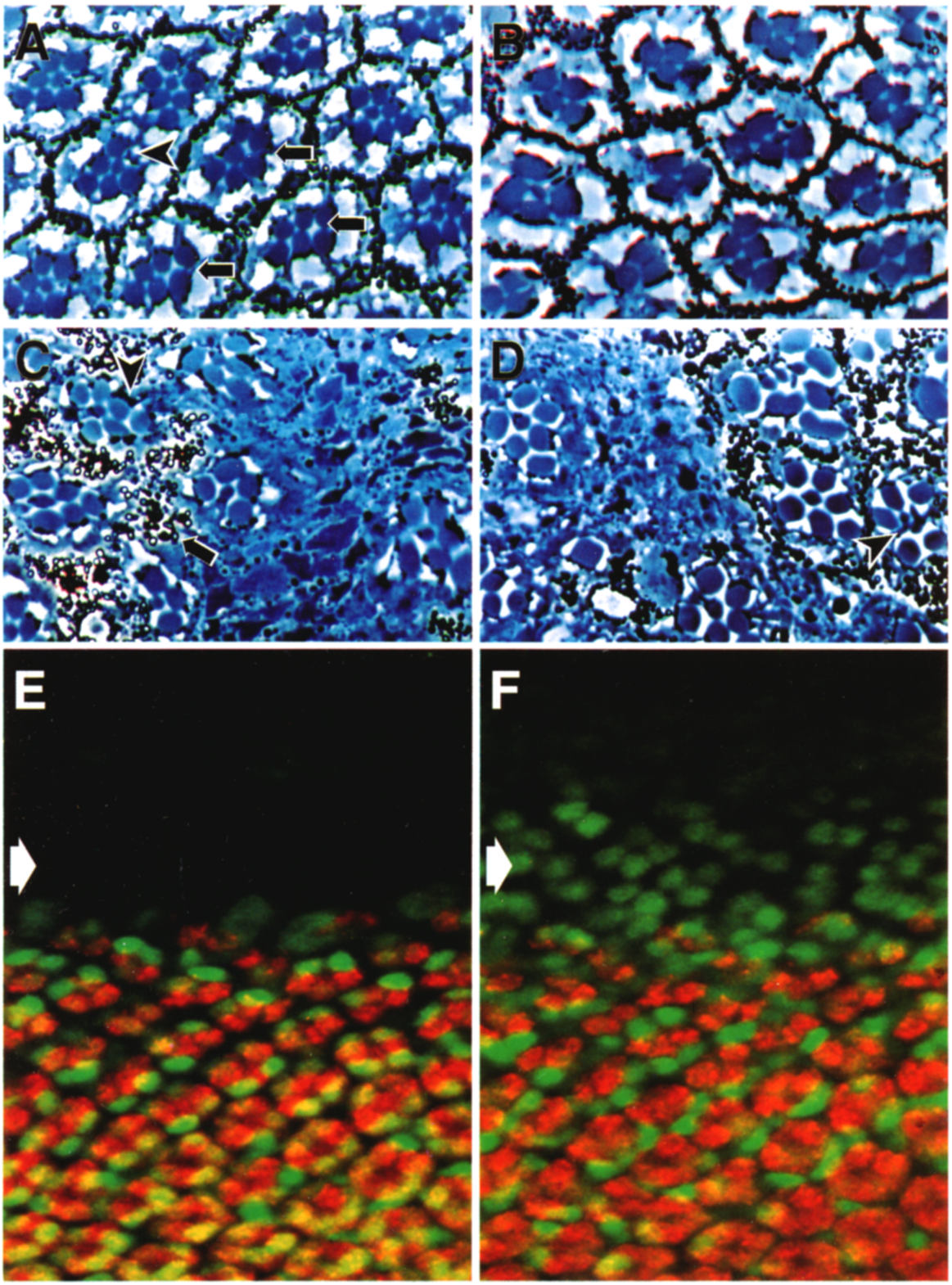
Among these lines, we identified four alleles of *pointed*, l(3)3520, l(3)3680, l(3)5484 and l(3)7825, which we found were missing photoreceptors in clones (data not shown). Transcription of the *pointed* gene is initiated from two promoters, called *P1* and *P2*, which drive the expression of two alternately spliced transcripts that code for two related proteins, referred to here as pointed^{P1} and pointed^{P2}

Figure 2. The Phenotype and Expression Pattern of *pointed* in the Eye

(A and B) The eyes of flies homozygous for hypomorphic alleles of *pointed* are missing photoreceptors. (A) A section through the eye of a fly homozygous for a weak hypomorphic allele of *pointed* (*pointed*$^{3680\Delta 21}$, an excision allele derived from line l(3)3680) is shown. The eyes of such flies have many wild-type ommatidial clusters, with only occasional missing photoreceptors, which are most commonly the R7 cell. Arrows indicate clusters that are missing the R7 cell, and the arrow head indicates a cluster that is missing an outer photoreceptor. (B) Flies homozygous for a stronger hypomorphic allele of *pointed* (*pointed*$^{7825\Delta 82}$, derived from l(3)7825) are missing multiple photoreceptors in almost every cluster. The R7 cell is missing in more than half of the clusters.

(C and D) *pointed* is required for cell fate determination in all photoreceptor subtypes and acts downstream of *Ras1* in a signal transduction pathway leading to photoreceptor determination. The FLP/FRT system for generating marked mitotic clones (Xu and Rubin, 1993) was used to generate small patches of *pointed*$^-$ tissue in the eyes of flies that were heterozygous for *pointed*$^{7825\Delta 78}$. (C) The fly that gave rise to the clone shown was otherwise genotypically wild type. (D) The clone shown was generated in a fly that also expressed activated Ras1 (chromosome CR2; see Experimental Procedures). Expression of activated Ras1 in this way causes the induction of ectopic R7 cells (Fortini et al., 1992). Several extra R7 cells are indicated by an arrow head. The product of the *white* gene was used as a cell-autonomous marker to label *pointed*$^+$ tissue; thus all *white*$^-$ cells are also *pointed*$^-$. Photoreceptor cells that express the *white* gene have small, dark pigment granules located near their rhabdomeres (arrow head, C). Other cells in the retina that express the *white* gene product produce larger refractory pigment granules (arrow, C). The area of *pointed*$^-$ tissue in each panel can be identified by the lack of refractory pigment granules in the accessory cells. In both cases, there are no *white*$^-$ photoreceptors observed. Near the border of each clone, there are clusters that are missing some rhabdomeres. In a few rare cases, we did observe *white*$^-$ (and therefore *pointed*$^-$) photoreceptors in clones generated using this allele. We estimate that such cells account for less than 1% of the *white*$^-$ cells that would have become photoreceptors in a wild-type background.

(E and F) The expression of pointed^{P2}. The P element in the enhancer trap allele *pointed*^{P3520} is inserted in the first exon of the *P2* transcript (see Figure 1). An eye imaginal disc from a third instar larva was double labeled with an antibody against β-galactosidase (shown in green) and an antibody against Elav (shown in red), a nuclear marker of neuronal differentiation in Drosophila. Strong staining in both channels appears yellow. Since the photoreceptor nuclei rise to the apical surface of the eye disc monolayer as they differentiate, Elav is only visible in apical focal planes.



The location of the center of the morphogenetic furrow is shown by an arrow in each panel. (E) An apical focal plane showing β-galactosidase (green) and Elav (red). Most, if not all, of the cells in this focal plane appear to express β-galactosidase. This includes photoreceptors and cone cells (data not shown). (F) The same apical focal plane showing Elav staining, overlaid with a basal focal plane showing β-galactosidase staining. β-Galactosidase is expressed beginning just anterior to the morphogenetic furrow, several hours prior to the time when neuronal differentiation occurs, and is present in the basal undifferentiated nuclei.

(Klämbt, 1993). Since not all of the *pointed* P alleles gave fully penetrant phenotypes in clones, we used imprecise excision of the P elements to generate stronger alleles. We also hoped to generate transcript-specific mutations that would allow us to determine the role for each of the two pointed proteins during eye development. A diagram of the genomic area containing the *pointed* gene with the location of the original P element insertions, the approximate genomic regions missing in a few selected excision alleles, and a diagram of the structures of the P1 and P2 proteins are shown in Figure 1.

We recovered excision events associated with each of the four P alleles that gave rise to fully wild-type revertants, semilethal mutants with rough eyes, and lethal mutations. The appearance of wild-type revertants demonstrates that the P elements in the original alleles were responsible for the mutant phenotype. Rough-eyed flies homozygous for weak semilethal alleles were found to have occasional missing photoreceptors, most commonly the R7 (Figure 2A), while stronger alleles caused the loss of 2–4 photoreceptors in most ommatidial clusters (Figure 2B).

To examine the eye phenotypes of selected lethal alleles, FRT chromosomes carrying these alleles were used to generate clones of homozygous mutant cells in adult eyes. Lethal excision lines derived from each of the four original P element insertions gave rise to clones with missing photoreceptors, although many lines, including all lines derived from l(3)3520, did not give fully penetrant phenotypes in clones. We were able to find multiple lethal excision alleles derived from lines l(3)3680, l(3)5484, and l(3)7825 that gave rise to clones with almost no *pointed*$^-$ photoreceptors, suggesting that *pointed* gene function is required in all photoreceptors (Figure 2C). We found alleles with this phenotype which, on a molecular level, appear to affect either the P1 or P2 protein only. Thus, we were unable to demonstrate a distinct role for each transcript during eye development.

We also examined marked mitotic clones of *pointed* in the third instar eye imaginal disc, at the time during development when photoreceptor determination occurs (data not shown). The *pointed*$^-$ cells in these clones almost never express *Elav*, an early marker of neuronal differentiation, suggesting that *pointed* is required for cell fate determination rather than simply being required at some later step of differentiation.

Expression of *pointed* in the Eye Imaginal Disc

Since synthesis of the two *pointed* transcripts is directed by two different promoters, we sought to examine the expression patterns of both pointed^{P1} and pointed^{P2}. Attempts to generate antibodies specific to each form of pointed have been unsuccessful to date. However, P element enhancer trap lines often express β-galactosidase in the same expression pattern as does the gene into which they are inserted. Two of our P alleles, l(3)3520 and l(3)7825, appear to be specific to the *P2* transcript, while the other two, l(3)3680 and l(3)5484, are inserted near the initiating ATG of the *P1* transcript. The two *P2* insertion lines express β-galactosidase in most, if not all, cells in the eye imaginal disc in and posterior to the morphogenetic furrow (Figures 2E and 2F). Significantly, expression is observed in cells several hours prior to the time when they begin to differentiate as neurons. Neither of the two insertions near *P1* coding sequences expresses β-galactosidase at detectable levels in the eye–antennal disc (data not shown). However, 3 out of the 16 *pointed* cDNAs we isolated from an eye–antennal disc cDNA library correspond to the *P1* transcript, suggesting that *P1* is expressed in the eye disc as well.

pointed Acts Downstream of *Ras1* in Eye Development

Having established that *pointed* is required for normal photoreceptor development, we wished to determine whether it might function downstream of *Ras1* during transduction of the signal that leads to photoreceptor determination. We therefore tested *pointed* in several genetic assays designed to identify genes required for transduction of the signal through Ras1.

Expression of an activated form of Ras1 under the control of the *sevenless* enhancer leads to the differentiation of ectopic R7 cells and roughening of the adult eye (Fortini et al., 1992). The phenotype of such eyes is very sensitive to the dose of genes required for transduction of the Ras1 signal and can be used as the basis of a dominant modifier screen aimed at identifying genes required for *Ras1* function (H. Chang, T Choi, F. Karim, M. Therrien, D. Wassarman, and G. M. R., unpublished data). As shown in Figure 3, loss-of-function alleles of *pointed* act as dominant suppressors of activated Ras1 in this assay, suggesting that *pointed* is a downstream effector of *Ras1* during photoreceptor determination.

To provide more evidence that *pointed* acts downstream of *Ras1*, we generated marked mitotic clones of *pointed*$^-$ tissue in the eyes of flies that also expressed activated Ras1 under the control of the *sevenless* enhancer/promoter (see Figure 2D). We observed almost no *pointed*$^-$ photoreceptors in such clones, as predicted if *pointed* acts downstream of *Ras1*.

To further analyze the role of *pointed* in the Ras1 signal transduction pathway, we examined genetic interactions between *pointed* and other known components of this pathway. Consistent with data found in other systems, mutations in the Drosophila homologs of Raf (*Draf*; Dickson et al., 1992) and MAP kinase kinase (*Dsor1*; Tsuda et al., 1993) have been found to be dominant suppressors of activated Ras1 (H. Chang, T Choi, F. Karim, M. Therrien, D. Wassarman, and G. M. R., unpublished data). *Draf*$^{HM\text{-}7}$ is a hypomorphic allele of *Draf* (Melnick et al., 1993). Hemizygous *Draf*$^{HM\text{-}7}$ males have slightly rough eyes that exhibit missing photoreceptor cells, most commonly the R7 (Melnick et al., 1993). *Dsor1*^{XS520} was isolated as an X-linked suppressor of activated Ras1 and has an eye phenotype similar to that of *Draf*$^{HM\text{-}7}$ (F. Karim and G. M. R., unpublished data). The phenotypes of hypomorphic alleles like *Dsor1*^{XS520} and *Draf*$^{HM\text{-}7}$ are often sensitive to changes in the dosage of genes whose products act elsewhere in the same pathway. As shown in Figure 3, halving the gene dosage of *pointed* enhances both the rough eye and missing photoreceptor phenotypes of *Dsor1*^{XS520} or *Draf*$^{HM\text{-}7}$.

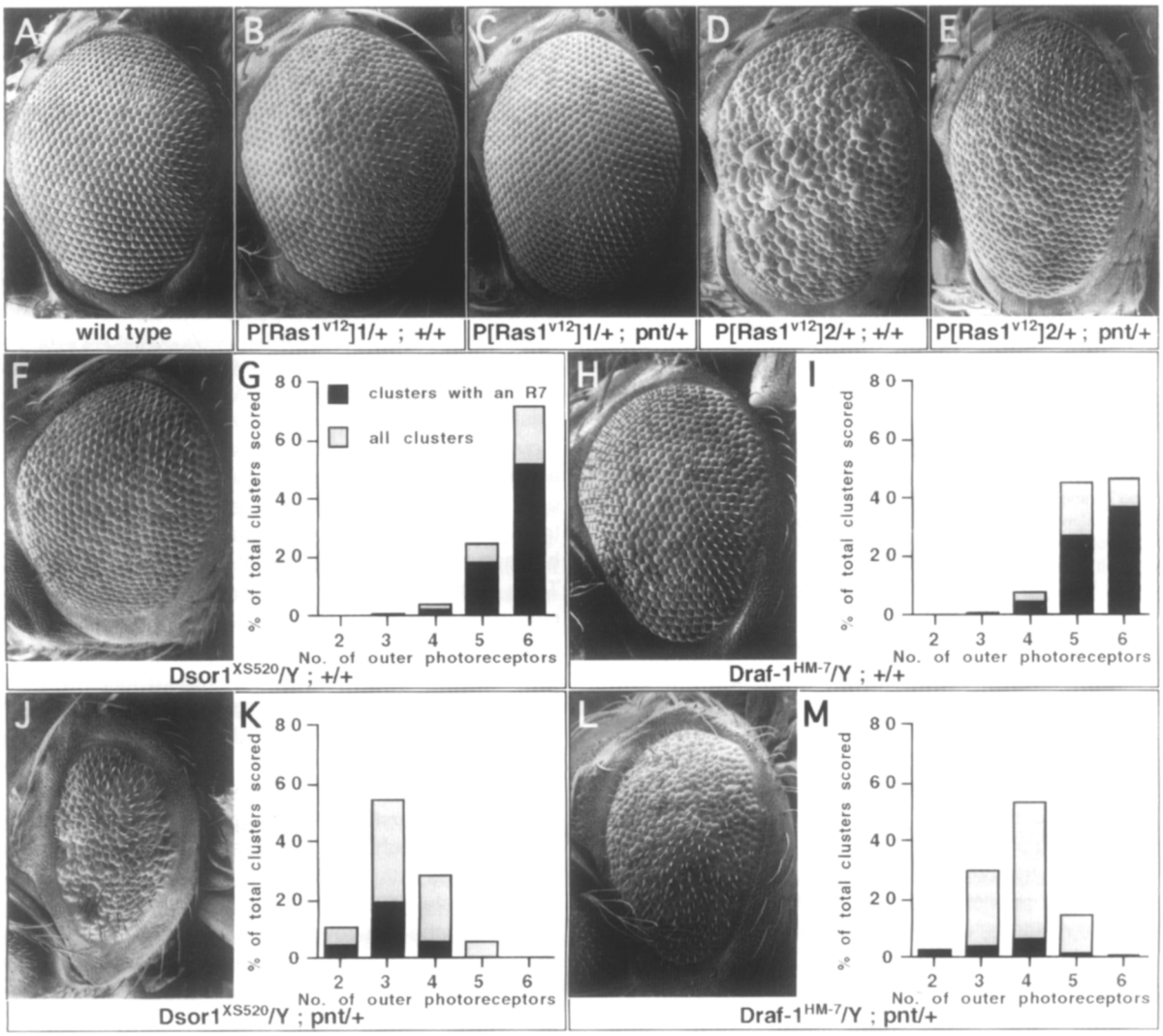


Figure 3. Genetic Interactions of *pointed* with Activated *Ras1* and Hypomorphic Alleles of *Draf* and *Dsor1*

(A) Scanning electron micrograph of the eye of a wild-type (Canton S) fly.

(B–E) *pointed* is a dominant suppressor of activated Ras1. Expression of activated Ras1 causes a disruption of the ommatidial array that results in roughening of the exterior eye morphology (see text). The two activated Ras1 lines used for this study exhibited either weak (line CR1, B) or moderate (line CR2, D) degrees of roughness. The eyes of flies carrying either the CR1 or the CR2 chromosome that are also heterozygous for *pointed* (C and E, respectively) have a more regular ommatidial array and fewer extra photoreceptors (data not shown) as compared with the eyes of flies wild type at the *pointed* locus.

(F, G, J, and K) *pointed* is a dominant enhancer of a hypomorphic allele of *Dsor1*, a gene encoding a MAPKK. (F) Flies hemizygous for $Dsor1^{XS520}$ have slightly rough eyes. The eyes of such flies were also sectioned and found to have occasional missing photoreceptors. (G) The phenotypes of 432 ommatidial clusters were scored both for the presence of an R7 cell and for the number of outer photoreceptors present. (J) Flies hemizygous for $Dsor1^{XS520}$ that are also heterozygous for *pointed* have much rougher eyes that are also reduced in size. (K) Both the missing R7 and missing outer photoreceptor phenotypes are enhanced (189 clusters scored).

(H, I, L, and M) *pointed* is a dominant enhancer of a hypomorphic allele of *Draf*. (H) Flies hemizygous for $Draf^{HM-7}$ (Melnick et al., 1993) have slightly rough eyes and (I) are missing occasional photoreceptors (556 clusters scored). (L) Flies hemizygous for $Draf^{HM-7}$ that are also heterozygous for *pointed* have much rougher eyes that are slightly reduced in size. (M) The missing photoreceptor phenotype of $Draf^{HM-7}$ is also dominantly enhanced by *pointed* (214 clusters scored).

The genotypes of flies shown in this figure are wild type (A); *P[sev-Ras1^{V12}]CR1/+; +/+* (B); *P[sev-Ras1^{V12}]CR1/+, pointed$^{7825\Delta 78}$/+* (C); *P[sev-Ras1^{V12}]CR2/+; +/+* (D); *P[sev-Ras1^{V12}]CR2/+; pointed$^{7825\Delta 78}$/+* (E); *Dsor1^{XS520}/Y; +/+* (F); *Draf^{HM-7}/Y; +/+* (H); *Dsor1^{XS520}/Y; pointed$^{7825\Delta 78}$/+* (J); and *Draf^{HM-7}/Y; pointed$^{7825\Delta 78}$/+* (L). The allele *pointed$^{7825\Delta 78}$* was derived from an imprecise excision screen beginning with the P allele *pointed^{P7825}*. On a molecular level, this allele appears to affect only DNA coding for the *P2* transcript (see Figure 1). However, it is possible that this excision also affects expression of the *P1* transcript. Multiple other excision alleles derived from *pointed^{P7825}* had similar, though somewhat weaker, effects. Excision events affecting *P1*-specific exons, including *pointed$^{5484\Delta 33}$* (derived from *pointed^{P5484}*), had effects in these assays similar to the weaker excision alleles derived from *pointed^{P7825}*. No excision events derived from *pointed^{P3520}* had any detectable effect in these assays. Molecular analysis of this class of excision event revealed that none were missing regions corresponding to coding sequences; thus, these alleles may not lack all *P2* function. There are two possible interpretations for the observed difference in the phenotypes between the two sets of excision alleles derived from the apparently *P2*-specific insertions *pointed^{P3520}* and *pointed^{P7825}*. Perhaps the lines derived from *pointed^{P3520}* are hypomorphs and the lines derived from *pointed^{P7825}* have lost all *P2* function, in which case a 50% reduction in *P2* gene dose is sufficient to cause an effect in these genetic assays. Alternatively, the *pointed^{P3520}* lines could represent true *P2* nulls, and a 50% reduction in *P2* gene function is not sufficient to cause an effect in our genetic assays. In this case, the *pointed^{P7825}* lines would have an effect in the genetic assays because they also disrupt *P1* function.

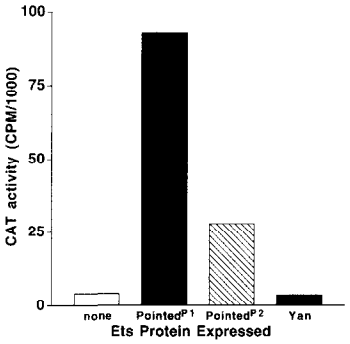


Figure 4. Transcriptional Activation Potential of Ets Proteins

A representative experiment showing a comparison of the transcriptional activation activities of the Ets proteins used in this study. When averaged over several experiments, the activation observed with pointed^{P1} (nine experiments) was 17.1 times the value observed for the vector alone control; with pointed^{P2} (seven experiments), 3.4 times the control value; and with yan (six experiments), CAT activity was always within 20% of the control value.

Activated Ras1 Up-Regulates the Transcriptional Activity of the P2 but Not the P1 Form of Pointed

Having established a role for *pointed* in the Ras1 pathway, we sought to examine the biochemical mechanism underlying the genetic interactions we observed. We began by testing the ability of each of the two forms of *pointed* to activate transcription in a cotransfection assay. A high-affinity binding site for Ets-1 has been identified using a binding site selection assay (Nye et al., 1992). Since the Ets domain of pointed is identical to that of Ets-1 at 95 out of 100 residues, we used this canonical binding site as a target of pointed in our assays.

We generated a reporter construct, E_6BCAT, consisting of six tandem copies of the high-affinity Ets site upstream of the *E1B* basal promoter followed by the bacterial chloramphenicol acetyltransferase (*CAT*) gene. Drosophila tissue culture cells were cotransfected with E_6BCAT and a second plasmid expressing either the *P1* or *P2* form of *pointed* under the control of the Drosophila actin *5C* promoter, and then cell lysates were assayed for CAT activity. We found that pointed^{P1} and pointed^{P2} each activated transcription, with pointed^{P1} being a significantly stronger activator than pointed^{P2} (Figure 4). This was the first piece of evidence we obtained showing a functional difference between the two forms of pointed.

Since our genetic data suggested that pointed activity might be stimulated by the Ras1 pathway, we looked directly at whether Ras1 could modulate the transcriptional activity of either form of pointed. This was accomplished using the same cell culture assay, except that we also cotransfected either the plasmid pPacRas1^{V12} or pPacRas1, which contain either an activated or the wild-type form of Ras1 under the control of the Drosophila actin *5C* promoter. Cotransfection of pPacRas1^{V12} increased pointed^{P2} activity about 5-fold (Figure 5A), whereas pPacRas1 stimulated its activity about 2-fold (data not shown). By contrast, we found that neither activated nor wild-type Ras1 had any effect on transcriptional activation by pointed^{P1}. Thus, in these transfection assays, we observe a significant difference in the regulation and activity of the two forms of pointed.

Pointed^{P2} May Be a Direct Target of ERKA

We next asked whether MAPK, which is known to function downstream of Ras, can regulate pointed^{P2} activity. A Drosophila MAPK called ERKA is encoded by the *rolled* locus. (Biggs and Zipursky, 1992; Biggs et al., 1994). Loss-of-function alleles of *ERKA/rolled* reduce signaling through the *sevenless* pathway (Biggs et al., 1994), while a gain-of-function allele of *ERKA/rolled*, called Sevenmaker or Sem, can bypass the requirement for *sevenless* and can cause the formation of extra R7 cells (Brunner et al., 1994). Since flies carrying this allele are viable, it is likely that this mutation results in only partial activation of MAPK. The molecular lesion that causes this phenotype is known (Brunner et al., 1994), so we introduced the appropriate change into the coding region of the *ERKA* gene and then tested its ability to modulate pointed activity in our transfection assay. We found that ERKA^{Sem} had the same qualitative

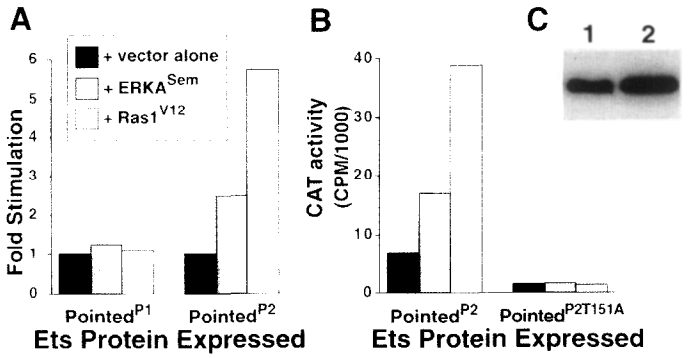


Figure 5. Ras1/MAPK-Dependent Effects on the Activity of Pointed^{P1}, Pointed^{P2}, and Pointed^{P2T151A}

(A) A representative experiment analyzing the stimulation of pointed^{P1} or pointed^{P2} by activated Ras1 or ERKA is shown. For each set of three samples, the fold stimulation shown is relative to the plus vector (+ vector) alone control. Stimulation of pointed^{P2} activity by ERKA^{Sem} and Ras1^{V12} averaged 2.3-fold and 4.7-fold, respectively, over seven experiments. The corresponding average values for pointed^{P1} were 1.06 and 0.99. Details of the calculation used to determine fold stimulation are described in Experimental Procedures.

(B) Mutating the putative phosphoacceptor residue in the MAPK consensus site of *P2* abolishes the ability of pointed^{P2} to activate transcription or respond to ERKA^{Sem} or Ras1^{V12}.

(C) An immunoblot of protein immunoprecipitated from cells transfected with a plasmid expressing a myc epitope-tagged version of pointed^{P2} (lane 1) or pointed^{P2T151A} (lane 2) demonstrates that both proteins are expressed at comparable levels. ERKA^{Sem} was coexpressed in the experiment shown. Similar results were obtained when the proteins were expressed alone or with Ras1^{V12}. The epitope-tagged versions of these proteins behave identically in our assays to the nontagged proteins.

effect as did activated Ras1 on pointed^{P2}, although the effect was somewhat weaker (Figure 5A). As was the case with Ras1^{V12}, ERKA^{Sem} had no effect on pointed^{P1} (Figure 5A). Overexpression of wild-type ERKA had no effect on either form of pointed (data not shown).

Examination of the amino acid sequence of the two forms of pointed for consensus MAPK phosphorylation sites (Gonzalez et al., 1991; Clark-Lewis et al., 1991) revealed a single consensus site in pointed^{P2} (see Figure 1). pointed^{P1} contains no consensus sites. We mutated the putative phosphoacceptor residue, Thr-151, in the pointed^{P2} consensus site to an alanine residue, and tested the activity of this mutant, *pointed*^{P2T151A}, in our assay. As shown in Figure 5B, the mutant protein has reduced activity on its own, but most importantly its activity is not stimulated by coexpression of activated Ras1 or ERKA. Immunoprecipitation from cells expressing the mutant protein indicate that it is expressed at levels comparable to wild type (Figure 5C). Moreover, gel mobility shift assays (data not shown) using extracts from transfected cells demonstrate that the mutant protein is still functional for DNA binding, though more rigorous studies will be necessary to determine whether there is a change in its affinity for DNA. These data suggest that the ability of pointed^{P2} to activate transcription is increased through direct phosphorylation by ERKA.

The activity obtained for pointed^{P2T151A} in this assay was much lower than that obtained for pointed^{P2}, even in the absence of added Ras1^{V12} or ERKA^{Sem}. One likely explanation for this result is that some fraction of pointed^{P2} becomes activated in these cells by an endogenous protein kinase. Indeed, a fraction of the endogenous ERKA in the immortalized cell line we use in these assays has been shown to be present in the activated form (Biggs and Zipursky, 1992). This basal level of activated ERKA might stimulate a fraction of the expressed pointed^{P2}, causing the weak transcriptional activation we observe when we transfect pPACpointed^{P2} alone, as in Figure 4. We believe that pointed^{P2} in its unphosphorylated form, by analogy to pointed^{P2T151A}, is probably unable to activate transcription at all.

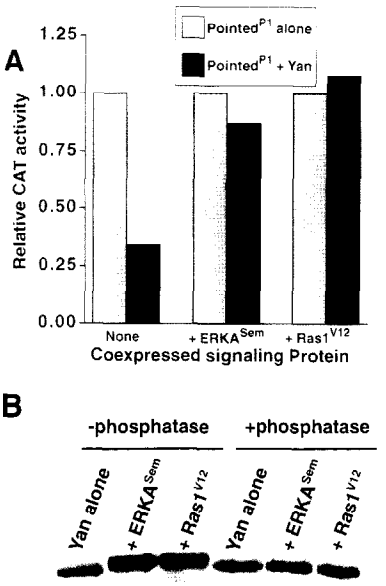


Figure 6. Regulation of Yan Repression and Phosphorylation by ERKA^{Sem} and Ras1^{V12}

(A) A representative experiment demonstrating the transcriptional repression activity of yan and its regulation by ERKA^{Sem} and Ras1^{V12} is shown. Within each pair of samples cotransfected with empty vector (none) or a plasmid expressing the indicated signaling protein (ERKA^{Sem} or Ras1^{V12}), relative CAT activity was calculated by dividing the activity obtained for each sample (with or without yan) by the activity observed within that pair by expression of pointed^{P1} without added yan. When averaged over several experiments, the values for pointed^{P1} + yan relative to pointed^{P1} were as follows: 0.29 for no added signaling protein (6 experiments), 0.76 for ERKA^{Sem} added (5 experiments), and 0.92 when Ras1^{V12} was added (6 experiments).

(B) An immunoblot of yan protein immunoprecipitated from cells transfected with pPACyan alone or in combination with a plasmid expressing either Ras1^{V12} or ERKA^{Sem}. Yan protein derived from cells that coexpress Ras1^{V12} or ERKA^{Sem} has a slower electrophoretic mobility than yan derived from cells that were transfected with pPACyan alone. Treatment of extracts with phosphatase after immunoprecipitation causes yan from cells cotransfected with pPAC Ras1^{V12} or pPACERKA^{Sem} to comigrate with yan from cells transfected with pPACyan alone. Extracts in lanes 4–6 were treated with calf intestinal phosphatase, while extracts in lanes 1–3 were mock treated.

Activated Ras1 and ERKA Negatively Regulate the Ability of Yan To Repress Transcription

yan, like *pointed*, encodes an Ets-related protein that functions genetically downstream of activated Ras1 during Drosophila eye development. However, in contrast with *pointed*, *yan* is required to negatively regulate photoreceptor determination, and flies homozygous for loss-of-function mutations of *yan* have extra R7 cells (Lai and Rubin, 1992; Tei et al., 1992). Although the Ets domain of yan is only 44% identical to that of human Ets-1 in the DNA-binding domain, yan protein derived from cells transfected with pPACyan can still bind to the canonical Ets site in a gel mobility shift assay (data not shown). However, when tested in the cotransfection assay, yan was unable to activate transcription through these sites (see Figure 4).

Since the genetic function of *yan* in photoreceptor determination is negative, a likely hypothesis is that yan acts biochemically to repress transcription. We therefore tested the ability of yan to repress the transcriptional activation activity of the constitutive, P1 form of pointed. As shown in Figure 6A, yan can repress the activity of pointed^{P1}. Cotransfection of pPACyan with pPACpointed^{P1} decreases pointed^{P1}-mediated transcriptional activation to an average of 29% of the value obtained in the absence of yan.

Genetic data suggest that the Ras1/MAPK pathway negatively regulates yan (Lai and Rubin, 1992), so we tested the effects of activated Ras1 and ERKA on yan in the cotransfection assay. Consistent with this genetic data, we found that the ability of yan to repress transcription is negatively regulated by activated Ras1 and ERKA (Figure 6A). Cotransfection of either pPACRas1^{V12} or pPACERKA^{Sem}, in addition to both pPACpointed^{P1} and pPACyan, alleviates yan-mediated repression of pointed^{P1} activity. Transcrip-

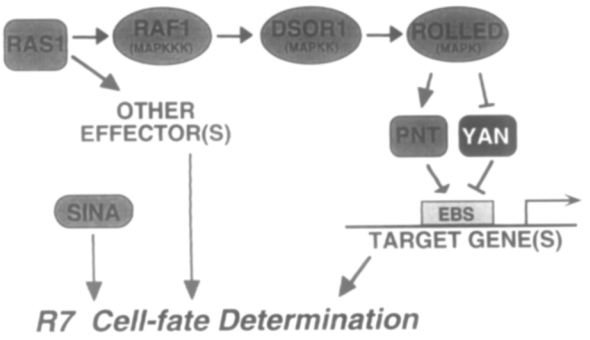


Figure 7. Model For the Role of *pointed* and *yan* in Photoreceptor Determination

The signal transduction cascade downstream of Ras1 during R7 cell determination is depicted. In the absence of a signal through Ras1, yan is active and represses the transcription, through EBS, of a set of genes that are required for R7 cell determination. Pointed^{P2} (PNT) is present, but not active, in the absence of a signal. In the presence of a signal through Ras1 and MAPK, pointed^{P2} becomes phosphorylated and activates transcription of its target genes through the EBS. Yan activity is down-regulated by the phosphorylation induced by the signal, and it is no longer capable of repressing transcription. If both pointed^{P2} and yan act through the same EBS, then yan could act to repress transcription by either a passive or an active mechanism. The Ets domains of pointed^{P2} and yan are only 44% identical, so they may bind to slightly different EBS in vivo. If they act through distinct EBS on the same promoters to regulate these genes, then yan must repress by an active mechanism. pointed is presumably regulated by one or more *sevenless*-independent, Ras1-mediated signal transduction cascade(s) during determination of the non-R7 photoreceptor subtypes. Sina is a nuclear protein that is required for R7 determination (Zipursky and Rubin, 1994). See text for alternative interpretations of the data.

tional activation is restored by expression of activated Ras1 and ERKA to an average of 92% or 76%, respectively, of the levels observed in the absence of yan.

Yan Is Phosphorylated in Response To Activated Ras1 and ERKA

The coding sequence of yan contains eight consensus MAPK phosphorylation sites, suggesting that yan might be directly phosphorylated in response to activation of the Ras1/MAPK pathway. Using polyclonal antiserum directed against the amino-terminal portion of yan, we immunoprecipitated yan from cells transfected with pPACyan, either alone or in combination with pPACRas1^{V12} or pPACERKA^{Sem}. As shown in Figure 6B, yan prepared from cells cotransfected with either pPACRas1^{V12} or pPACERKA^{Sem} had a slower electrophoretic mobility than did yan from cells transfected with pPACyan alone. This altered mobility appears to be due to phosphorylation of yan since treatment of the extracts with phosphatase causes the more slowly migrating forms to comigrate with yan derived from cells transfected with pPACyan alone. Thus, there is an inverse correlation between the phosphorylation state and repressor activity of yan, suggesting that yan is negatively regulated by phosphorylation.

Discussion

We have demonstrated that *pointed* gene function is required downstream of the Ras1/MAPK pathway for photoreceptor determination during Drosophila eye development. We have described the positive regulation of the transcriptional activator pointed^{P2} by Ras1 and ERKA, and we have shown that the P1 form of pointed is a constitutively strong transcriptional activator that is unresponsive to Ras1/MAPK signaling. Moreover, we have presented evidence for the negative regulation of the repressor activity of yan by the Ras/MAPK pathway. These data, taken together with previously known information, lead us to suggest a model for the role of these Ets-related transcription factors in eye development (Figure 7). In this model, we propose the existence of a group of genes required for photoreceptor determination whose expression is controlled through EBSs. In the absence of a signal through MAPK, the yan protein is present and active and therefore represses transcription. When signals instructing a cell to become a photoreceptor are transmitted through MAPK from the cell surface, yan is down-regulated and pointed^{P2} is stimulated. Transcription of target genes is initiated, and photoreceptor differentiation begins. While we cannot rule out other interpretations, this model is the simplest one consistent with our data.

How Does the Ras/MAPK Pathway Modulate the Activity of Pointed^{P2} and Yan?

We have shown both that the activity of pointed^{P2} is increased in response to the Ras/MAPK pathway and that mutation of the single MAPK consensus phosphorylation site in pointed^{P2} abrogates this responsiveness. In combination with our genetic data, this strongly suggests that ERKA directly phosphorylates pointed^{P2}, stimulating its activity. Since there are precedents for similar phosphorylation events stimulating the activity of several other transcription factors (Hibi et al., 1993; Gonzalez and Montminy, 1989), including an Ets protein stimulated by MAPK (Marais et al., 1993; Janknecht et al., 1993), we believe that this direct phosphorylation model is the most likely mechanism by which pointed^{P2} becomes activated in our assay. However, we cannot formally eliminate other possibilities, including the existence of another kinase that acts between ERKA and pointed^{P2} or the direct phosphorylation of some other protein that is required for pointed^{P2} activity and that cannot act when Thr-151 is mutated.

We have shown that the Ras1/MAPK pathway acts to phosphorylate and negatively regulate yan. Downregulation of either DNA binding or transcriptional repression activity are two possible mechanisms by which phosphorylation might negatively regulate yan. Other possibilities include alteration of the stability or subcellular localization of the yan protein.

The Mechanism of Yan-Mediated Repression

We have demonstrated the ability of yan to repress pointed^{P1}-mediated transcriptional activation. Many mechanisms of transcriptional repression have been described (reviewed by Herschbach and Johnson, 1993). In the assay we use to measure repression, both the activator and the repressor bind to the same sites. This leaves open the possibility that yan represses transcription by a passive mechanism involving competition with pointed^{P1} for the EBSs. Thus,

the reduced activation we observe could be due solely to the fact that yan occupies the EBS, thereby preventing pointed^{P1} from activating transcription. Alternatively, yan might contain an active repression domain. Future work will be necessary to distinguish between these two mechanisms.

Other interpretations of the repression activity of yan observed in our assay are possible as well. For example, yan could function in vivo as an activator of transcription in combination with another factor missing from the cultured cells used in our assays. Perhaps in the cotransfection assay, yan passively represses transcription, even though in vivo yan could turn on a distinct set of genes that negatively regulate photoreceptor determination. Nevertheless, we have established that yan activity is regulated by the Ras1/MAPK pathway.

Is the *P1* Form of *pointed* Regulated by the Ras/MAPK Pathway?

Although the activity of pointed^{P1} protein itself does not appear to be modulated by the Ras1/MAPK pathway, it is possible that the *P1* form of the *pointed* gene is regulated by another mechanism. One appealing model is that activated pointed^{P2} might act to turn on the *P1* promoter. Since pointed^{P2} is probably active only transiently, when the Ras1 signal is present, this form of positive autoregulation would allow a prolonged EBS-activating signal in the cell. The idea that pointed^{P2} might exert its effect through activation of the *P1* promoter is consistent with the fact that we were unable to identify transcript-specific mutations with distinct phenotypes. This idea is also consistent with the fact that *pointed* alleles affecting only pointed^{P1} (on a molecular level) act as dominant modifiers in our genetic assays; perhaps the *P1* promoter is responsive to the Ras/MAPK pathway, even though the activity of the pointed^{P1} protein is not regulated.

Signal Integration during Drosophila Eye Development

Cells in the developing retina receive multiple signals from neighboring cells during development. In addition to the positive signals cells receive, they also receive signals through the *Notch* gene product that delay or negatively influence photoreceptor fate determination in the retina (Fortini et al., 1993). Moreover, other as yet unidentified signaling events probably take place as well. These signals must all be integrated by the undetermined cells in the developing eye in such a way that proper identity can be assessed in each cell. Since alteration of gene expression is the ultimate mechanism by which cell fate is established in each cell, a likely integration point for these signals is at the level of posttranslational modification of transcription factor activity. Transcription factors other than Ets-related proteins are probably involved in this process as well. It is also possible that there are multiple, temporally distinct signals mediated by MAPK during photoreceptor determination and that the activities of yan and pointed^{P2} are modified in response to distinct signals.

Ras1, *drk*, and *Son of sevenless*, like *pointed*, are required for the determination of all photoreceptor subtypes (Simon et al., 1991), indicating that there are non-*sevenless*-mediated signals upstream of *Ras1* in cell types other than R7. One candidate for such a receptor is the Drosophila homolog of the epidermal growth factor receptor or Egfr. *Egfr* gene function is required in all photoreceptors (Xu and Rubin, 1993). The mammalian EGFR has been shown to signal via a Ras-dependent pathway (reviewed by Schlessinger, 1993), and genetic interactions have been observed between alleles of *Egfr* and *Ras1* (Simon et al., 1991). Further work will be required to identify the receptor(s) acting upstream of *pointed* in each photoreceptor subtype.

The observation that there are extra R7 cells present in *yan* mutant flies, even when the *sevenless*-mediated MAPK signal is also lacking (Lai and Rubin, 1992), suggests that MAPK-dependent activation of pointed^{P2} might not be essential for photoreceptor determination. However, it is possible that MAPK is activated in these cells through a second, *sevenless*-independent signaling pathway and that MAPK activation of pointed^{P2} is still required for the precursor cells to differentiate as neurons. *Egfr* might mediate such a second signal.

An alternative model for the appearance of these extra cells does not require MAPK activation of pointed^{P2}. Whether or not a cell becomes a photoreceptor is normally determined by the balance of positive and negative signals received by that cell. Perhaps in the complete absence of *yan*, positively acting factors other than pointed^{P2} are sufficient to cause neural differentiation. By contrast, during normal development, while yan activity might be downregulated but not eliminated, activated pointed^{P2} would be required to overcome a low level of yan repression and drive cells into the neural pathway.

Concluding Remarks

We have demonstrated that two Ets-related proteins function downstream of MAPK in signal transduction cascades, leading to photoreceptor determination in the Drosophila eye. One protein is a repressor negatively regulated by the pathway, and the other is an activator stimulated by the pathway. Both proteins are likely to be direct substrates of MAPK, and they likely provide a link between activation of the sevenless RTK at the cell surface and changes in gene expression in the nucleus.

Experimental Procedures

Genetics

The collection of lethal P element insertion lines was generated by Karpen and Spradling, (1992), and the cytological locations of the P insertions were mapped by the Drosophila Genome Project (Berkeley, California). The cytological position of *pointed* is 94F1–2. Excision alleles of *pointed* were derived from four original P alleles. P elements were excised by providing a stable source of transposase and then by selecting individual *rosy*$^-$ males.

CR1 and CR2 are each CyO chromosomes that carry a P element expressing activated Ras1 under the control of the *sevenless* enhancer/promoter (Fortini et al., 1992; H. Chang and G. M. R. unpublished data). Crosses involving *Raf*$^{F^{HM-7}}$ were performed at 18°C.

Molecular Biology

pointed cDNAs were isolated from an eye–antennal disc cDNA library constructed by Dr. A. Cowman. Of 16 cDNAs isolated using a *pointed*^{P2}

cDNA as a probe (Chen et al., 1992), 3 corresponded to the *P1* form, 10 were derived from the *P2* form, and 3 were truncated, containing only sequences common to both forms. Plasmids were generated as described in O'Neill (1994).

Transfections and CAT Assays

Transfections were performed essentially as described (Pascal and Tjian, 1991). In all transfections, 100 ng of each indicated expression plasmid was cotransfected along with 2 μg of E_6BCAT and 3 μg of pUC118 by the calcium phosphate method. Within each experiment, the total amount of expression vector transfected into each plate was the same, with pPACUbx+NdeI vector alone used to make up for differences between samples with different numbers of proteins expressed. In each experiment, 2–4 plates were transfected in parallel with the same plasmids, and the resulting data were averaged. Time courses of CAT activity were performed as described (Neumann et al., 1987). A plot of each time course was generated, and a single time point from the linear portion of each curve was used to compare samples to each other.

In Figure 5A, fold stimulation was determined for each set of three samples by first subtracting the vector background appropriate for each sample (i. e., empty pPACUbx+NdeI vector either alone or cotransfected with a plasmid expressing $Ras1^{V12}$ or $ERKA^{Sem}$). This value was then divided by the activity obtained by cotransfection of the indicated Ets plasmid with empty vector (again, minus vector alone background).

Cotransfection of plasmids expressing activated Ras1 or ERKA in the absence of a plasmid expressing an exogenous Ets protein caused weak stimulation of transcription by E_6BCAT, possibly owing to activation of endogenous Ets proteins. This effect was small, and we corrected for it by subtracting these background values in all experiments as described. For example, in Figure 5A, the original CAT activities (in counts per minute) were 4606 for pPACUbx+NdeI vector alone, 5976 for pPACUbx+NdeI plus $pPACERKA^{Sem}$, and 8105 for pPACUbx+NdeI plus $pPACRas1^{V12}$; these were compared with values of 11387 for $pPACpointed^{P2}$ alone, 22930 for $pPACpointed^{P2}$ plus $pPACERKA^{Sem}$, and 47020 for $pPACpointed^{P2}$ plus $pPACRas1^{V12}$.

Immunoprecipitations and Phosphatase Treatment

For the experiments shown in Figures 5C and 6B, Drosophila S2 cells were transfected as described (Diederich et al., 1994), using 8 μg of plasmid expressing the indicated Ets protein either alone or with 2 μg of $pPACERKA^{Sem}$ or $pPACRas1^{V12}$, as indicated. Immunoprecipitations were performed essentially as described (Fehon et al., 1990), except that protein G–Sepharose beads (GammaBind Plus, Pharmacia) were used. Also, for the experiment shown in Figure 5C, the monoclonal antibody 9E10 was cross-linked to GammaBind Plus, and no secondary antibody was added. Phosphatase reactions were performed as described (Kaffman et al., 1994). The samples were then loaded onto a 7.5% denaturing polyacrylamide gel and immunoblotted according to standard procedures. Monoclonal antibody 9E10 (Figure 5C) or anti-yan polyclonal antiserum (Figure 6B) was used for the primary antibody, and enhanced chemiluminesence (ECL, Amersham) was used to visualize bands.

Acknowledgments

This manuscript was improved by comments from Jessica Treisman and Grace Gill. Kelli Lopardo provided assistance with cell culture, and Gina Dailey assisted with molecular biology. The authors thank Tanya Wolff for assistance with microscopy, Felix Karim for generating some of the *ERKA* constructs, and Erin O'Shea and members of the Rubin lab for helpful discussions. I. R. is a fellow of the Life Sciences Research Foundation. This work was partially supported by a National Institutes of Health grant (GM33135) to G. M. R. R. T. and G. M. R. are investigators with the Howard Hughes Medical Institute.

Received April 20, 1994; revised June 8, 1994.

References

Biggs, W. H., III, and Zipursky, S. L. (1992). Primary structure, expression, and signal-dependent tyrosine phosphorylation of a *Drosophila* homolog of extracellular signal-regulated kinase. Proc. Natl. Acad. Sci. USA *89*, 6295–6299.

Biggs, W. H., Zavitz, K. H., Dickson, B., van der Straten, A., Brunner, D., Hafen, E., and Zipursky, S. L. (1994). The *Drosophila rolled* locus encodes a MAP kinase required in the Sevenless signal transduction pathway. EMBO J. *13*, 1628–1635.

Brunner, D., Oellers, N., Szabad, J., Biggs, W. H., III, Zipursky, S. L., and Hafen, E. (1994). A gain of function mutation in Drosophila MAP kinase activates multiple receptor tyrosine kinase signaling pathways. Cell *76*, 875–888.

Chen, T., Bunting, M., Karim, F. D., and Thummel, C. S. (1992). Isolation and characterization of five *Drosophila* genes that encode an ets-related DNA binding domain. Dev. Biol. *151*, 176–191.

Chen, R. H., Abate, C., and Blenis, J. (1993). Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. Proc. Natl. Acad. Sci. USA *90*, 10952–10956.

Clark-Lewis, I., Sanghera, J. S., and Pelech, S. L. (1991). Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase. J. Biol. Chem. *266*, 15180–15184.

Conrad, K. E., Oberwetter, J. M., Vaillancourt, R., Johnson, G. L., and Gutierrez-Hartmann, A. (1994). Identification of the functional components of the Ras signaling pathway regulating pituitary cell-specific gene expression. Mol. Cell. Biol. *14*, 1553–1565.

Dickson, B., Sprenger, F., Morrison, D., and Hafen, E. (1992). Raf functions downstram of Ras1 in the Sevenless signal transduction pathway. Nature *360*, 600–603.

Diederich, R. J., Matsuno, K., Hing, H., and Artavanis-Tsakonas, S. (1994). Cytosolic interaction between deltex and notch ankyrin repeats implicates deltex in the notch signaling pathway. Development *120*, 473–481.

Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M. A. T., and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci *Notch* and *Delta*, two EGF-homologous genes in Drosophila. Cell *61*, 523–534.

Fortini, M. E., Simon, M. A., and Rubin, G. M. (1992). Signalling by the sevenless protein tyrosine kinase is mimicked by Ras1 activation. Nature *355*, 559–561.

Fortini, M. E., Rebay, I., Caron, L. A., and Artavanis-Tsakonas, S. (1993). An activated Notch receptor blocks cell-fate commitment in the developing *Drosophila* eye. Nature *365*, 555–557.

Gonzalez, G. A., and Montminy, M. R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell *59*, 675–680.

Gonzalez, F. A., Raden, D. L., and Davis, R. J. (1991). Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. J. Biol. Chem. *266*, 22159–22163.

Herschbach, B. M., and Johnson, A. D. (1993). Transcriptional repression in eukaryotes. Annu. Rev. Cell Biol. *9*, 479–509.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. *7*, 2135–2148.

Janknecht, R., Ernst, W. H., Pingoud, V., and Nordheim, A. (1993). Activation of ternary complex factor Elk-1 by MAP kinases. EMBO J. *12*, 5097–5104.

Kaffman, A., Herskowitz, I., Tjian, R., and O'Shea, E. K. (1994). Phosphorylation of the transcription factor PHO4 by a cyclin–CDK complex, PHO80-PHO85. Science *263*, 1153–1156.

Karpen, G. H., and Spradling, A. C. (1992). Analysis of subtelomeric heterochromatin in the *Drosophila* minichromosome Dp1187 by single P element insertional mutagenesis. Genetics *132*, 737–753.

Klämbt, C. (1993). The *Drosophila* gene *pointed* encodes two ETS-like proteins which are involved in the development of the midline glial cells. Development *117*, 163–176.

Lai, Z.-C., and Rubin, G. M. (1992). Negative control of photoreceptor development in Drosophila by the product of the *yan* gene, an ETS domain protein. Cell *70*, 609–620.

Marais, R., Wynne, J., and Treisman, R. (1993). The SRF accessory protein Elk-1 contains a growth factor–regulated transcriptional activation domain. Cell *73*, 381–393.

Marshall, C. J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Genet. Dev. *4*, 82–89.

Melnick, M. B., Perkins, L. A., Lee, M., Ambrosio, L., and Perrimon, N. (1993). Developmental and molecular characterization of mutations in the *Drosophila*-raf serine/threonine protein kinase. Development *118*, 127–138.

Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T., and Akira, S. (1993). Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. Proc. Natl. Acad. Sci. USA *90*, 2207–2211.

Neumann, J. R., Morency, C. A., and Russian, K. O. (1987). A novel rapid assay for chloramphenicol acetyltransferase gene expression. Biotechniques *5*, 444–447.

Nye, J. A., Petersen, J. M., Gunther, C. V., Jonsen, M. D., and Graves, B. J. (1992). Interaction of murine ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. Genes Dev. *6*, 975–990.

O'Neill, E. M. (1994). Ph. D. thesis, University of California at Berkeley, Berkeley, California.

Pascal, E., and Tjian, R. (1991). Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. Genes Dev. *5*, 1646–1656.

Schlessinger, J. (1993). How receptor tyrosine kinases activate Ras. Trends Biochem. Sci. *18*, 273–275.

Scholz, H., Deatrick, J., Klaes, A., and Klämbt, C. (1993). Genetic dissection of *pointed*, a *Drosophila* gene encoding two ETS-related proteins. Genetics *135*, 455–468.

Simon, M. A., Bowtell, D. D. L., Dodson, G. S., Laverty, T. R., and Rubin, G. M. (1991). Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. Cell *67*, 701–716.

Tei, H., Nihonmatsu, I., Yokokura, T., Ueda, R., Sano, Y., Okuda, T., Sato, K., Hirata, K., Fujita, S. C., and Yamamoto, D. (1992). *pokkuri*, A *Drosophila* gene encoding an E-26-specific (Ets) domain protein, prevents overproduction of the R7 photoreceptor. Proc. Natl. Acad. Sci. USA *89*, 6856–6860.

Treisman, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. Curr. Opin. Genet. Dev. *4*, 96–101.

Tsuda, L., Inoue, Y. H., Yoo, M.-A., Mizuno, M., Hata, M., Lim, Y.-M., Adachi-Yamada, T., Ryo, H., Masamune, Y., and Nishida, Y. (1993). A protein kinase similar to MAP kinase activator acts downstream of the raf kinase in Drosophila. Cell *72*, 407–414.

Wasylyk, C., Gutman, A., Nicholson, R., and Wasylyk, B. (1991). The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. EMBO J. *10*, 1127–1134.

Wasylyk, B., Hahn, S. L., and Giovane, A. (1993). The Ets family of transcription factors. Eur. J. Biochem. *211*, 7–18.

Xu, T., and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development *117*, 1223–1237.

Zipursky, S. L., and Rubin, G. M. (1994). Determination of neuronal cell fate: lessons from the R7 neuron of *Drosophila*. Annu. Rev. Neurosci. *17*, 373–397.