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Abstract

Cell fate in the developing eye is determined by a cascade of inductive interactions. In this process, the sevenless protein—a receptor tyrosine kinase—is required for the specification of the R7 photoreceptor cell fate. We have constructed a gain-of-function sevenless mutation (SevS11) by overexpressing a truncated sevenless protein in the cells where sevenless is normally expressed. In SevS11 mutant flies, all sevenless-expressing cells initiate neural development. This results in the formation of multiple R7-like photoreceptors per ommatidium. Therefore, sevenless activity appears to be necessary and sufficient for the determination of R7 cell fate. These results illustrate the central role receptor tyrosine kinases can play in the specification of cell fate during development.
Ligand-Independent Activation of the sevenless Receptor Tyrosine Kinase Changes the Fate of Cells in the Developing Drosophila Eye

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Introduction

During the development of multicellular organisms, cells adopt distinct fates in response to cues in their environment. Little is known about the mechanisms by which cells read and interpret these cues and how the specificity is achieved by which neighboring cells reproducibly choose different developmental pathways. The formation of the R7 photoreceptor cell in the Drosophila eye is a model system to study mechanisms of position-dependent cell fate determination: an undetermined precursor cell is recruited to differentiate into a UV-sensitive photoreceptor cell based on its position with respect to its neighbors (reviewed in Basler and Hafen, 1988a; Rubin, 1989; Banerjee and Zipursky, 1990). Its presence can be assessed using morphological and behavioral criteria.

The R7 cell is part of the precise array of eight photoreceptor cells (R1 to R8) that, together with 12 nonneuronal cells, constitute an ommatidial unit. During development the ommatidial cluster assembles in a stereotyped sequence (Figure 1): the R8 cell is the first to differentiate, followed by the pairwise addition of R2/R5, R3/R4, R1/R6, and, finally, R7 (Tomlinson and Ready, 1987a). Each cell forms a unique set of cell contacts. The R7 precursor contacts the developing R1, R6, and R8 cells. Since no cell lineage relationships are shared by the different cell types in the eye (Lawrence and Green, 1979), it has been proposed that cell fate is determined by local cell–cell interactions (Ready et al., 1976; Tomlinson and Ready, 1987a).

Two mutations, bride of sevenless (boss) and sevenless, have been identified in which the R7 cell fails to develop correctly and instead becomes a nonneuronal cone cell. The presence of the R7 cell is caused by changing the fate of ommatidial precursor cells. These results indicate that sevenless activity is sufficient for the specification of R7 cell fate and are consistent with a model in which R7 cell fate is determined by the spatially and temporally restricted expression of the ligand for sevenless on R8.

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sevenless protein lacking most of its extracellular domain.

In previous attempts to obtain an activated sevenless receptor we introduced point mutations and C-terminal truncations in the sevenless coding region, hoping to imitate structural changes associated with transforming tyrosine kinases. None of the resulting products seemed to have a dominant effect in vivo (unpublished data). Another attempt to obtain a dominant phenotype by elevating the expression of sevenless involved the expression of a sevenless cDNA under the control of the inducible hsp70 heat shock promoter (Basler and Hafen, 1989a). We found, however, that the levels of sevenless protein produced per cell upon heat shock induction were lower than the amount of sevenless protein produced under the control of the sevenless enhancer. We therefore searched for alternative ways to achieve overexpression of sevenless.

Overexpression of a Truncated sevenless Protein (sev-S11) by Duplication of the sevenless Enhancer Sequences

The sequences required for normal sevenless expression have properties of a transcriptional enhancer (Basler and Hafen, 1989a). We therefore reasoned that levels of sevenless protein could be elevated by duplicating the enhancer sequences. To investigate the effect of enhancer duplication on the level of gene expression, we used the dosage-dependent misexpression of the rough gene as a test system (Basler et al., 1990). We found that the blueberry eye phenotype associated with transforming oncogene products indicate that constitutive activation of receptor tyrosine kinases can be caused by overexpression and/or structural alterations (for review see Jarden and Ullrich, 1988). In previous attempts to obtain an activated sevenless receptor we introduced point mutations and C-terminal truncations in the sevenless coding region, hoping to imitate structural changes associated with transforming tyrosine kinases. None of the resulting products seemed to have a dominant effect in vivo (unpublished data). Another attempt to obtain a dominant phenotype by elevating the expression of sevenless involved the expression of a sevenless cDNA under the control of the inducible hsp70 heat shock promoter (Basler and Hafen, 1989a). We found, however, that the levels of sevenless protein produced per cell upon heat shock induction were lower than the amount of sevenless protein produced under the control of the sevenless enhancer. We therefore searched for alternative ways to achieve overexpression of sevenless.

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The P element construct we assembled (sev-S11) is out lined in Figure 2. It contains sevenless sequences corresponding to amino acids 1865-2554 (Basler and Hafen, 1988b) under the transcriptional control of the duplicated sevenless enhancer and the hsp70 promoter. To ensure correct membrane topology, we included sequences derived from the Drosophila cuticle gene CP3 (Snyder et al., 1992) to provide an N-terminal signal sequence.

There are at least three possible outcomes of the transformation experiment with the sev-S11 construct using a sevenless mutant strain as a recipient. First, if the sev-S11 construct produces an inactive sevenless protein, we would observe the sevenless mutant phenotype. Second, if the sev-S11 construct indeed results in ligand-independent sevenless tyrosine kinase activity, but sevenless kinase activity is not sufficient to induce R7 development, then only the H7 precursor would develop into an H7' cell, and we would observe rescue of the sevenless mutant phenotype. Finally, if the sev-S11 protein is constitutively active and activation of the sevenless kinase is sufficient to induce R7 development in all the cells where it is expressed, we should observe the formation of multiple R7 cells.

The sev-S11 Construct Causes a Dominant Rough Eye Phenotype That Is Dosage Dependent and Requires a Functional sevenless Kinase

Two independent transformed lines (Sev217') were obtained that, in contrast to the recipient strain, exhibit a striking rough eye phenotype (Figure 3, left and middle). The same phenotype was observed when Sev217 was crossed into a sevenless background. Therefore, Sev217 is the first gain-of-function mutation known in the sevenless gene. The following characterization of the dominant Sev217 phenotype was carried out in a sev217 mutant background. To determine whether this dominant phenotype was caused by the increased expression of the truncated sevenless protein due to the duplicated sevenless enhancer, we made a variant of the sev-S11 construct that contained only one copy of the sevenless enhancer. Transformants of this construct showed the rough eye phenotype only when they were homozygous for the insert (data not shown). Therefore, the Sev217 phenotype is dependent on the amount of sev-S11 protein produced.

To test whether this dominant rough eye phenotype was caused by ectopic activity of the sevenless kinase or...
whether it was due to the mere abundance of the truncated protein, we constructed a mutant sev-S11 construct (sev-S11LysMet; Figure 2) with a single Lys to Met amino acid change at position 2242. This lysine residue is part of the ATP-binding site of the tyrosine kinase domain. We have previously shown that this amino acid substitution in the wild-type sevenless gene produces an inactive sevenless protein unable to determine R7 cell fate (Basler and Hafen, 1988b). Transformants containing one or two copies of the sev-S11LysMet construct did not exhibit any roughening of the eye (Figure 3, right). Therefore the dominant rough eye phenotype observed in SevS\textsuperscript{+} transformants is dependent on a functional tyrosine kinase.

Sev\textsuperscript{S11} Ommatidia Have Additional Photoreceptor Cells with Small Rhabdomeres

Each of the eight photoreceptor cells in a wild-type ommatidium has a microvillar stack of membranes containing rhodopsin, called the rhabdomeres. The rhabdome regions of the R1 to R6 photoreceptors form an asymmetric trapezoid and span the depth of the retina. The rhabdome of the R7 cell is smaller in diameter and occupies a central position in the distal half of the retina (Figures 4A and 4E). Since some rhabdomeres are fused or split, it is difficult to determine the exact number of photoreceptor cells per ommatidium. Examination of 163 ommatidia in four eyes revealed an average of six cells with large rhabdomeres and four cells with small rhabdomeres. Many ommatidia were found that contained up to seven small rhabdomeres in addition to six or seven large ones. In sections through the proximal region of the retina of Sev\textsuperscript{S11} transformants only one small rhabdomere cell corresponding to the R8 photoreceptor was detected in each ommatidium (data not presented).
Figure 4. sev<sup>−11</sup> Causes the Formation of Multiple Photoreceptors with Small Rhododemes

Histological sections through wild-type (A), sev<sup>−11</sup> (B), sev<sup>−12</sup>, Sev<sup>−11</sup> (C), and sev<sup>−2</sup> sev<sup>−11</sup><sup>−12</sup> (D) eyes are shown, as well as enlargements of single ommatidial units of wild type (E), sev<sup>−11</sup> (F), and sev<sup>−2</sup> Sev<sup>−11</sup> (G). Each photoreceptor cell has a microvillar stack of membranes containing rhodopsin, termed rhabdomere, that projects toward the center of the ommatidium. The R7 rhabdomere differs morphologically from the rhabdomeres of R1 to R6. It is smaller in diameter and occupies a central position in the wild type (see numbering in [E]). In sevenless the R7 cell is missing (B and F). In Sev<sup>−11</sup> there are on average more than six photoreceptor cells visible in each ommatidium; many have small rhabdomeres (C and G). In sev<sup>−11</sup><sup>−12</sup>, as in the sevenless recipient, only six photoreceptor cells are visible (D). Anterior is to the right. Magnification is 1000 x for (A) through (D) and 2400 x for (E) through (G).

shown). Thus the sev-S11 construct results in the recruitment of multiple photoreceptors with small rhabdomeres in a sevenless mutant background. The morphological characteristics of the additional photoreceptor cells with small rhabdomeres and occupying a distal portion of the retina resemble those of R7 cells.

The Multiple Small Rhabdomere Cells in Sev<sup>−11</sup>

Ommatidia Are Functional R7-like Photoreceptor Cells

To test whether the small rhabdomere cells are indeed R7 cells, we crossed the sev-S11 construct into an ora (outer rhabdomeres absent) mutant background. ora mutants
lack rhodopsin in the R1–6 set of photoreceptors; in addition, this mutation causes the selective degeneration of the rhabdomeres of the outer photoreceptor cells H1 to R6 (Stark and Sapp, 1987). Ommatidia of flies homozygous for ora in an otherwise wild-type background possess only R7 and R8 rhabdomeres (Figure 5A). A section through the distal part of an eye of a Sev<sup>571</sup>; ora double mutant is shown in Figure 5B. On average, there are four small rhabdomeres per ommatidium as in the ora<sup>+</sup> background. In the proximal part of the Sev<sup>571</sup> retina only the

Figure 5. The Photoreceptor Cells with Small Rhabdomeres in Sev<sup>571</sup> Are Differentiated R7 Photoreceptor Cells

(A–C) Histological sections through the distal part of the retinas of ora flies (A) and ora; Sev<sup>571</sup> flies (B) and through the proximal portion of ora; Sev<sup>571</sup> eyes (C). The multiple small rhabdomere cells in Sev<sup>571</sup> do not degenerate in the ora background (B). In proximal sections through the retina of ora; Sev<sup>571</sup> flies only a single rhabdomere (R6) is present (C).

(D–F) Frozen head sections stained for β-galactosidase activity of sevenless<sup>+</sup>; Rh3-β-gal (D), sevenless<sup>−</sup>; Rh3-β-gal (E), and Sev<sup>571</sup>; Rh3-β-gal (F). The Rh3-β-gal construct is expressed in a subpopulation of H7 cells in wild type (D); no enzymatic activity is detected in the retina of sevenless flies since they lack R7 cells. In Sev<sup>571</sup> (F), many more cells express the Rh3-β-gal construct than in wild type. These cells are therefore fully differentiated R7 photoreceptors.

Magnification is 1000 x in (A) through (C) and 200 x in (D) through (F).
Cell  
1074

\[ \lambda (\text{GREEN/UV}) = \frac{n(\text{GREEN}) - n(\text{UV})}{\text{TOTAL}} \]

Figure 6. Color Choice Preference of Wild-Type (Canton S), sev^2, and sev^2; Sev^511 flies in a T-Maze

Flies were tested for the color choice preference between 360 nm UV light and 550 nm green light. The phototactic value \( \lambda \) represented graphically was determined as indicated at the top of the figure. Flies from the sev^2 strain that served as recipients for injection are attracted by the green light since they lack R7 cells. In contrast, sev^2, Sev^511 flies are strongly attracted by the UV light, indicating that at least some of the multiple R7 cells are functional and make proper connections in the medulla of the optic lobes.

R8 rhabdomere is present (Figure 5C). The finding that the multiple small rhabdomeres in Sev^511 transformants do not degenerate in the ora mutant is consistent with the assumption that these cells are R7 photoreceptors.

The only biochemical markers of fully differentiated R7 cells distinguishing them from other photoreceptor cells are the R7-specific rhodopsins Rh3 and Rh4 (Zuker et al., 1987; Monte11 et al., 1987; Fortini and Rubin, 1990). They are expressed in nonoverlapping, complementary subsets of the R7 photoreceptor cell populations. To assay for the presence of fully differentiated R7 cells in Sev^511, we generated a transformant strain containing the bacterial \(/\alpha cZ\) gene driven by the \(\&\)-regulatory sequences of the Rh3 rhodopsin gene (Rh3-p-\(\&\)-gal). Whereas a random subpopulation of R7 cells express this Rh3-p-\(\&\)-gal fusion gene in a wild-type background (Figure 5D), no staining is observed in sevenless mutant flies because they lack R7 cells (Figure 5E). In Sev^511 transformants many more cells express the Rh3-p-\(\&\)-gal construct than in wild type. Thus, many of the cells with small rhabdomeres of Sev^511 transformants express the R7-specific rhodopsin Rh3 and therefore have properties of fully differentiated R7 cells.

To examine whether the R7-like cells formed in Sev^511 transformants are also functional and make correct synaptic connections, we tested the phototactic behavior of Sev^511 flies. The R7 photoreceptor cells are responsible for the attraction of flies to UV light when given a choice between that and green light. Flies lacking R7 photoreceptors such as the sev^2 recipients are attracted preferentially toward the green light (Harris et al., 1976). The data in Figure 6 show that sev^2, Sev^511 flies are strongly attracted to UV light, indicating that at least some of the R7 cells make functional synaptic connections.

We conclude that the multiple photoreceptor cells with small rhabdomeres in Sev^511 ommatidia are supernumerary R7-like cells. The evidence is several-fold: First, the size of the rhabdomeres and their position in the distal part of the retina are morphological characteristics of R7 cells. Second, the fact that they do not degenerate in an ora background indicates that they are not R1-6 cells. Third, expression of the Rh3-p-\(\&\)-gal marker by many cells in the Sev^511 transformants further indicates that these cells have properties of fully differentiated R7 cells. Fourth, the behavioral test demonstrates that at least some of these R7-like cells are functional and make correct connections in the medulla of the optic lobes.

The Formation of R7-like Cells in Sev^511 Is Not Dependent on boss

An important question is whether the formation of R7-like cells in Sev^511 is dependent on the boss gene. The boss gene product has been shown to be required in the R8 cell for R7 development to occur (Reinke and Zipursky, 1988). Recently, the boss gene was cloned and sequenced. It encodes a membrane protein with a large extracellular domain (Hart et al., 1990). It is possible that the boss gene product may itself be the ligand for the sevenless receptor. In Figure 7A, a section through a Sev^511; boss mutant fly is shown. In the boss mutant background we observe on average the same number of R7-like cells as in a boss+ background (Figure 7A). Thus, the recruitment of multiple
ommatidial precursors into the R7 developmental pathway in Sev^{s''} appears to be independent of the boss gene.

All Cells Expressing sev-S11 Initiate Neuronal Development

To identify the origin of the multiple R7-like cells in Sev^{s''}, we examined the assembly of the ommatidial clusters in the imaginal discs of sevenless mutants and Sev^{s''} transformants. We suspected that the R7-like cells would be recruited from the ommatidial precursor cells that express the sev-S11 construct. Using a polyclonal antiserum directed against the sevenless kinase domain, we confirmed that the truncated sev-S11 protein is expressed in the same subset of ommatidial precursor cells as the wild-type protein (Figure 8). Both proteins are transiently expressed at high levels in R3 and R4, in one or two of the so-called mystery cells, in R7, and, finally, in the cone cells. Only weak expression is seen in R1 and R6 (Tomlinson et al., 1987). To follow the assembly of the ommatidial cluster, we used the monoclonal antibody BP-104. This antibody recognizes a neuron-specific form of the Drosophila neuroglian protein and serves as an early differentiation marker for neuronal cells in Drosophila (Hortsch et al., 1990). We examined optical sections through whole-mount preparations of BP-104-stained eye imaginal discs of sevenless and Sev^{s''} larvae. We found that in Sev^{s''} discs all cells that express sev-S11 also express BP-104 and therefore initiate neuronal development. This is shown in Figure 9 and described in detail below.
Figure 9. All Cells That Express sevenless Initiate Neural Development in Sev\textsuperscript{617}

Optical sections of whole-mount eye imaginal discs that have been stained with the neural-specific monoclonal antibody BP-104. Low magnification view is shown of a sevenless disc (A) and a Sev\textsuperscript{617} disc (F). Anterior is to the right. The temporal sequence of ommatidial assembly is spatially displayed along the anterior-posterior axis. The rightmost column corresponds to column 3 in (A) and column 5 in (F). High magnification views are shown of selected stages of ommatidial assembly in a sevenless disc (B-E) and a Sev\textsuperscript{617} disc (G-K). During disc development, the ommatidia undergo a 90° rotation. All clusters are shown in their final orientation such that anterior is to the right. In sevenless, five cells corresponding to R2, R3, R4, R5, and R6 are stained in column 5 (B). In Sev\textsuperscript{617}, one to two additional cells, m1 and m2, are visible between R3 and R4 (G). These cells correspond to the mystery cells that normally express sevenless but do not initiate neuronal development in wild type or in sevenless mutants. In column 9, behind the morphogenetic furrow, photoreceptors R1 and R6 but not R7 have initiated differentiation in sevenless (C). In Sev\textsuperscript{617}, both the R7 cell between R1 and R6 and a mystery cell between R3 and R4 express the BP-104 antigen (H). In column 11 the photoreceptor cell cluster has moved basally such that only the apical projections of the photoreceptor cells are visible in sevenless (D). The unstained cell between R1 and R6 visible at this stage (arrowhead in [D]) corresponds to the R7 precursor that does not initiate neuronal development in sevenless but becomes a cone cell instead. The presumptive cone cells come to lie above the photoreceptor cells but are unstained in sevenless mutants or in wild type. At the same stage in Sev\textsuperscript{617}, however, we observe in addition to the stained R7 cell three cone cells that are also stained with the BP-104 antibody (I). In column 13 only the microvillar processes of the photoreceptors are visible in the apical section in sevenless (E). In Sev\textsuperscript{617}, the four presumptive cone cells are visible because they express the BP-104 neural marker (K). Abbreviations: 1-6, photoreceptor cells R1 to R6; m, mystery cells; c, cone cells. Magnification is 630 x in (A) and (F) and 1260 x in (B) through (E) and (G) through (K).

However, the R7 cell is visible between R1 and R6 in addition to one of the mystery cells between R3 and R4 (Figure 9H). After the integration of all the photoreceptor cells has occurred, these cells move basally in the epithelium, and the cone cells will come to lie above the photoreceptor cell cluster. Therefore in wild type and in sevenless only the apical projections of the photoreceptors can be seen in an apical section through the disc (Figure 9A). Since the cone cells do not initiate neuronal development normally they are not visible in BP-104-stained discs. In sevenless one unstained cell is located between cells R1 and R6 (Figure 9D, arrowhead). This cell corresponds to the R7 precursor that has assumed a cone cell identity and remains apically in the cluster. In Sev\textsuperscript{617}, however, not only the R7 cell but also the presumptive anterior, posterior, and polar cone cells stain strongly with BP-104 (Figure 9I).

In column 14 and 15 of sevenless discs only the extreme apical projections of the photoreceptors are visible (Figure 9E). In Sev\textsuperscript{617}, rosettes are detectable apically consisting of four to five cells that correspond to the presumptive cone cells and the R7 cell (Figure 9K). We conclude that in Sev\textsuperscript{617} all cells that express sevenless during ommatidial assembly can initiate neuronal development. In comparison to the sevenless strain, six to seven additional cells express the BP-104 antigen: one or two mystery cells, the R7 precursor, and the four presumptive cone cells. This is the maximum number of BP-104-positive cells per ommatidium and is therefore somewhat higher than the average of four R7-like cells observed in the Sev\textsuperscript{617} retina. In 12% of the ommatidia, however, we do observe six to seven R7-like cells. Cells R3 and R4, which also express sevenless, initiate neuronal development also in sevenless...
Activation of sevenless Alters Cell Fate

Figure 10. Cobalt Sulfide Staining of Wild-Type and SevS" Pupal Discs

Eye imaginal discs of 67 hr wild-type (A) and SevS" (B) pupae were stained with cobalt sulfide to outline the pattern of cone cells and pigment cells. In wild type, the four cone cells are surrounded by two primary pigment cells. These clusters are embedded in a hexagonal pattern of secondary and tertiary pigment cells. Although the pattern in SevS" is less regular, up to five cone cells are visible surrounded by the pigment cells. This suggests additional cells have been recruited as cone cells in SevS". Anterior is to the right. Magnification is 2000 x.

mutants. Since in SevS" retinas we detect on average six large rhabdomere cells, it seems likely that R3 and R4 do not become R7 cells.

The spatial and temporal expression pattern of sevenless has been analyzed in detail only in larval discs. Integration of the primary, secondary, and tertiary pigment cells, however, occurs after puparium formation. To determine whether sevenless is expressed in any of these cells, we stained eye discs of 40 hr pupae with sevenless antisera. Staining with the sevenless antiserum was confined to the cone cells in the anteriormost clusters (data not shown). None of the pigment cells showed detectable levels of sevenless protein. We conclude, therefore, that all cells that express sev-S11 can initiate neural development.

Additional Cells Are Recruited in SevS" Flies to Substitute for the Neuronalized Cone Cell Precursors

If all the cells including the presumptive cone cells that secrete the lens initiate neuronal development in SevS", which cells will form the ommatidial lenses? Using cobalt sulfide staining to outline the cell boundaries, we examined the later stages of ommatidial assembly in discs of wild-type and SevS" 67 hr pupae. In wild type, the four cone cells are surrounded by the two primary pigment cells (Figure 10A). The secondary and tertiary pigment cells including the bristle cells form a lattice around the ommatidial units. Although the pattern in the SevS" disc is less regular (Figure 10B), each ommatidial unit has an average four cone cells surrounded by primary pigment cells. Staining of pupal discs with the BP-104 antibody indicates that the cells surrounded by the primary pigment cells are indeed nonneuronal (data not shown). It therefore appears that additional cells are recruited in SevS" to become cone cells after the original cone cell precursors have been subverted into neuronal development. It is possible that the variable number of cells we count in the SevS" retina is due to a general shortage of precursor cells to complete all ommatidial units. Consistent with this interpretation is the observation that the SevS" eyes consist of about 15% fewer lens-bearing ommatidia than sevenless or wild-type eyes (Figure 3).

Discussion

We have previously shown that sevenless tyrosine kinase activity is necessary for the determination of the R7 photoreceptor cell (Basler and Hafen, 1988b). To determine whether tyrosine kinase activity of sevenless is sufficient to direct other ommatidial precursor cells into the R7 developmental pathway, we generated a sevenless allele (SevS") that produces a constitutively active protein. A dominant phenotype is associated with SevS", and our analysis shows that it is caused by recruitment of supernumerary R7-like photoreceptors.

Overexpression of a Truncated sevenless Protein Leads to Ligand-Independent Activation of the sevenless Kinase

Two types of mechanisms have been described that can account for ligand-independent activation of receptor tyrosine kinases. First, structural alterations, in particular point mutations affecting the extracellular domain or the transmembrane region, have been described for the products of the neu and the v-fms proto-oncogenes (Bargmann et al., 1986; Woolford et al., 1988; Roussel et al., 1988). These mutations are believed to induce a conformational change favoring receptor dimerization equivalent to that triggered by ligand binding. Second, in contrast, many abnormalities involving growth factor receptors in human malignancies appear to involve the overexpression of unaltered proteins. For example, it has been shown that overexpression alone can activate the erbB-2 gene as a transforming oncogene in tissue culture cells (Di Fiore et al., 1987). An increased concentration of a receptor tyrosine kinase may cause transformation by raising the level of constitutive kinase activity to a threshold required for growth stimulation.

The sev-S11 protein is missing a large portion of its extracellular domain. This structural alteration might partly relax the normal dependence of sevenless activity on li-
gand binding. We favor the alternative explanation that the truncation primarily leads to increased amounts of tyrosine kinase-bearing protein. This is supported by the observation that its effect is dosage dependent and that it seems to be several-fold more efficiently expressed upon heat shock induction compared with full-length protein (unpublished data). The biological effect appears to be a direct measure of sevenless tyrosine kinase domain concentration. The dependence on an intact ATP-binding site indicates that it is not the mere presence of the sevenless tyrosine kinase but rather its activity that accounts for the Sev<sup>571</sup> phenotype.

Several lines of evidence indicate that the effect of Sev<sup>571</sup> is ligand independent: First, additional R7-like cells are recruited in positions where in wild type sevenless is expressed but no R7 cells are generated. Since expression of the putative ligand for sevenless is not altered in our experiment, this effect must be independent of it. Second, the same effect is observed in a boss<sup>−/−</sup> background, boss being the most likely candidate for a ligand for sevenless. Third, although the ligand-binding region of sevenless is unknown, sev-S11 lacks 88% of its extracellular domain, rendering it unlikely to be able to functionally interact with its ligand. More recent results with chimeric receptors containing only the C-terminal intracellular part of the sevenless protein, however, lead to the same activation (D. Dickson and E. Hafen, unpublished data), excluding the possibility that the short extracellular domain of sev-S11 is responsible for the activation of the kinase domain by binding to a ligand.

**Constitutive Tyrosine Kinase Signaling:**

**Determination versus Proliferation**

Constitutive activation of receptor tyrosine kinases has been studied so far primarily by virtue of their transforming capacity in tissue culture cells or by their oncogenic potential in tumor formation. The common pattern in both cases is uncontrolled cell proliferation. Although, in principle, the multiple R7-like cells in Sev<sup>571</sup> could have arisen by induced proliferation of the R7 precursor, our developmental analysis can exclude this possibility. Neural antigens are detected in the mystery cells of Sev<sup>571</sup> discs approximately 8 hr before the R7 precursor enters the ommatidial cluster. Thus, these cells deviate from their normal developmental pathway prior to R7 determination and therefore cannot be descendants of the R7 precursor.

Therefore, in contrast to the elevated tyrosine kinase activity of a growth hormone receptor, constitutive activation of sevenless does not lead to the proliferation of cells but to the transformation of cell fate. This difference reflects the unequal role of the signals these receptors mediate normally: proliferation as a response to a growth factor versus cell fate determination upon interaction with an inductive cue. Cells normally not exposed to the positional signals as proposed by the combinatorial model (Tomlinson and Ready, 1989). Thus in many cells, sevenless activity appears to be necessary and sufficient for, and not merely a prerequisite or temporal trigger of, R7 development.

In this respect, the role of sevenless in cell fate determination is different from that of Notch. Notch is also required for the cell–cell interactions that control cell fate decisions in the eye. Removal of Notch activity at different steps of ommatidial assembly results in cells selecting inappropriate developmental pathways (Cagan and Ready, 1989). Whereas Notch is required but seems to play a permissive role, sevenless is not required for the formation of the terminal anlagen of the embryo (Sprenger et al., 1989). The torso protein is expressed in all the cells of the blastoderm but is activated only locally at the poles (Casanova and Struhl, 1989). Ec-topic activation of torso in the dominant alleles causes a change in cell fate of the central cells of the blastoderm. Instead of forming the segmented parts of the body, these cells differentiate into terminal structures. As in the case of sevenless, activation of the receptor causes a cell fate change rather than the onset of proliferation.
role in the determination of cell fate, sevenless activity appears to have an instructive role.

Predictions about the Distribution of the Ligand for sevenless

Since wild-type cone cell precursors do not develop into R7 cells although they express sevenless, the ligand for sevenless cannot be freely diffusible but must be spatially restricted, most likely to the developing R8 cell. The mystery cells, however, also contact R8 initially and do not initiate neural development normally, although they can if sevenless is activated. This suggests that the sevenless ligand is not seen by the mystery cells, most likely because it is expressed late in R8 development at the time when only the R7 cell is ready to be determined. Thus, a combined spatial and temporal restriction in the expression of the ligand for sevenless seems to specify that only the R7 precursor cell will respond to it.

Our observation that sevenless activity is sufficient to specify R7 cell fate suggests that there is only a single signaling pathway to control R7 induction. It therefore strongly supports the hypothesis that boss and sevenless act in the same pathway. Since the boss gene encodes a membrane protein with a large extracellular domain (Hart et al., 1990), it is possible that the boss protein is the membrane-bound ligand for sevenless on the R8 cell.

Experimental Procedures

DNA Constructs

sev-S11 and sev-S11LysMet

The P element vector used for transformation is based on pW8 (Klemenz et al., 1987) bearing the selectable marker gene white. A duplication of the sevenless enhancer was obtained by cloning the 1.2 kb genomic sevenless XhoI fragment (positions 6347-7564) into pBluescript that was linearized with EcoRI and phosphatase treated. A clone was selected containing two copies of the XhoI fragment in the same orientation as shown in Figure 2. The sevenless cDNA insert was then excised and replaced with a shorter and modified cDNA fragment (see below) to give rise to sev-S11.

The sequences encoding a truncated sevenless protein with an exogenous signal peptide were obtained as follows: Two partially complementary oligonucleotides with the sequences 5'TCCTTCTGGACCCA-ACATGTCAAGATCTGCTTGTCCTCTGGCCGGCGCCGGTGGG-CCGCCAACGGCATTAGCTACC-3' and 5'CGGCCCGCCTCAGTCGCTTCTCCTGAGATACCTGCTTGTCCTCTGGCCGGCGCCGGCG-ACC-3' were annealed and extended to obtain a double-stranded DNA fragment coding for the amino acid sequence MKILLVCSSLAALVANRSEQKLISEEDLNGR. This sequence represents the signal peptide of the Drosophila cuticle protein CP3 (Snyder et al., 1982) followed by a 10 amino acid stretch of a c-myc epitope for which a monoclonal antibody (9ElO) is available (Evan et al., 1985; Munro and Pelham, 1987). The fragment was cloned, sequenced, and then joined with its 3' Ndel site to the pCIa1 site (genomic position 12325) of the sevenless cDNA, resulting in an in-frame fusion to amino acids 1885-2554 of the sevenless protein. The cDNA used in this construction is fused in the same orientation. Using adjacent sites in the pBluescript polylinker, this tandem enhancer was isolated, blunt ended, and inserted into the filled-in EcoRI site of hsp-sev cDNA type A (see Basler et al., 1989) such that it was placed in front of the hsp70 promoter/leader in an appropriate orientation as shown in Figure 2. The sevenless cDNA insert was then excised and replaced with a shorter and modified cDNA fragment (see below) to give rise to sev-S11.

The hsp-rough fusion gene was excised from pW8[hsp-rough] with EcoRI and PstI and subcloned into derivatives of pBluescript that contained either a duplicated 1.2 kb sevenless enhancer (see above) or only a single copy in either orientation. The three resulting constructs were excised with Apal (filled in with T4 polymerase) and XbaI and inserted into pDM23 (containing the rosy marker gene; Miser and Rubin, 1987) digested with Apal (filled in with T4 polymerase) and XbaI. The two orientations of the single enhancer constructs gave rise to an identical phenotype as sev-hsp-rough (Basler et al., 1990).

The genomic 0.5 kb HindIII fragment of Rn3 (Zuker et al., 1987) was subcloned into pBluescript KS+ and the promoter fragment was amplified by the polymerase chain reaction using the universal M13 (~20) primer and the oligonucleotide 5'CAGGATCCGGTCTGGCCGCCG 3' (corresponding to positions 6 to 26). A BamHI site was thereby introduced between the transcription and the translation initiation site. The reaction product was digested with BamHI and cloned into pUCM/v (Miser and Rubin, 1987), resulting in a translational fusion to the lacZ gene.

Drosophila Strains and Germline Transformation

Plasmid DNA was prepared for injection as described previously (Basler and Hafen, 1988) except that we used pUCM/v 23 as helper plasmid (gift of Don Rio). Embryos of the w t°°, sev^+ genotype were injected with sev-S11LysMet. The chromosomal site of integration of the sev-S11 construct was determined by st in situ hybridization as described by Basler and Hafen (1988). Sev-S11 maps to position 18Q on the X chromosome, and sev-S11.5 to position 75C on the left arm of the third chromosome. For the experiments described here the X-linked line sev-S11.4 was used. Five independent sev-S11LysMet lines were obtained. For the analysis described here, the X linked line sev-S11LysMet was used. The sev-hsp-rough construct and the Rn3-b-gal construct were injected into the y w^ host strain.

Scanning Electron Microscopy and Histology

Adult flies used for scanning electron microscopy were stored in 70% acetone before they were critical-point dried and coated to be examined in a Hitachi S-4000 scanning electron microscope. Eyes to be sectioned were fixed and embedded in Spurr's medium as described previously (Basler and Hafen, 1988). Semithin sections (1 μm) were obtained and stained with toluidine blue for light microscopy.

Cobalt Sulfide Staining of Pupal Discs

Staining was done essentially as described by Melamed and Trujillo-Cenoz (1975). Briefly, pupae were dissected in Ringer's solution and the eye discs were transferred for 5 min to 2% glutaraldehyde in phosphate-buffered saline (PBS). The discs were washed in distilled water for 30 s and then incubated in 2% Co(NO₃)₃, for 5 min. The discs
were washed again in distilled water for 5 s and then transferred to 1% (NH₄)₂SO₄ for 15 s. The discs were washed for several minutes in distilled water and mounted in DABCO (1,4-diazabicyclo[2.2.2]octane (2.5% in 87% glycerol). The age of the pupae refers to their development at 20°C.

β-Galactosidase Staining
Eyes were embedded in OCT compound freezing medium (Miles Inc.), and 8 μm sections were cut with the cryostat. Sections were mounted on gelatinized slides and incubated at 60°C for 1 min. The sections were then fixed in 1% glutaraldehyde in PBS for 15 min, washed three times for 10 min in PBS, and rinsed in staining buffer (Simon et al., 1989). Afterward the sections were incubated in staining solution overnight at 37°C. Sections were washed in PBS for 10 min, air dried, and mounted in CMCP-10 (Polysciences, Inc).

Immunohistochemistry and Confocal Microscopy
Eye imaginal discs were dissected in Ringer's and then fixed for 30 min in 4% paraformaldehyde in 0.1 M phosphate buffer, 0.1 M glucose, and 0.07 M CaCl₂. The discs were washed in PBS, 0.2% saponin for 30 min. During this step the peripodial membrane was removed from those discs that were subsequently stained with the anti-sevenless antiserum to increase the sensitivity of signal detection. The discs were then fixed in 30 min in blocking solution (1 x PBS, 0.2% saponin, 3% fetal calf serum; Sigma). Incubation in the primary antibody was usually done overnight at 4°C. The discs were washed three times for 5 min in PBS, 0.2% saponin. The FITC-conjugated secondary antibodies (Southern Biotechnologies, Inc.) were used at a 1:50 dilution in blocking solution. Incubation was done for 2 hr at room temperature. After three successive 5 min washes in PBS, 0.2% saponin, the discs were mounted in DABCO (2.5% in 87% glycerol).

For the analysis of the expression of truncated sev-Sll protein in eye disc, we used the affinity-purified polyclonal goat antisera G24, which was raised against a bacterial fusion protein corresponding to the C-terminal cytoplasmic domain of the sevenless protein. To examine the assembly of the ommatidial cluster in sevenless and Sev-Sll eye imaginal discs where stained with monoclonal antibody BP-104 (kindly provided by Nipam Patel and Corey Goodman). The BP-104 monoclonal supernatant was diluted 1:1 in blocking solution (PBS, 3% fetal calf serum, 0.2% saponin). The discs were examined first by conventional fluorescence microscopy to select specimens that were mounted flat. These discs were then examined using the Bio-Rad confocal system MRC-600 connected to a Zeiss Axiohot with a 4× planapo chromatom lens (Zeiss). Serial optical sections (Z-series) at 0.5 μm intervals were obtained. For the analysis of the ommatidial clusters, single sections approximately 2 μm below the apical surface of the discs were used. Photographs were obtained directly from the computer monitor using IBM HP Laser Plus film. For the low magnification views shown in Figure 8 and Figure 9, prints of optical sections of different positions on the same disc were spliced together.

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