tramtrack is a transcriptional repressor required for cell fate determination in the Drosophila eye

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Cell fate determination in the Drosophila eye is mediated by inductive events between neighboring cells in the eye imaginal disc. These inductive signals lead to differential gene expression necessary for the elaboration of different cell types in the compound eye. Several putative transcription factors have been identified previously that may be required for expression of genes that specify cell fate in the compound eye. Repression of inappropriate gene expression may be as important as transcriptional activation in the determination of cell fate. We report the identification of a mutation in the Drosophila tramtrack (ttk) locus that is required for cell fate determination in the compound eye. ttk is expressed as two proteins, p69 and p88, shown previously to bind to the regulatory regions of several segmentation genes. In ttk¹, an allele missing the mRNA encoding p88, many ommatidia contained supernumerary R7 cells and decreased numbers of RI–R6 cells. ttk len, which appears to disrupt both Ttk proteins, was characterized by early embryonic arrest as well as transformation of ommatidial cells into nonommatidial cell types in mosaic flies. Consistent with previous proposals that the Ttk proteins are transcriptional repressors of segmentation genes, we detected ectopic or increased expression of the segment polarity gene engrailed in several ttk¹ larval tissues. We propose that p69 is required to repress expression of genes that are incompatible with development of photoreceptor cell fates, whereas p88 appears to be required to repress genes that promote the R7 cell fate.

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Cell fate specification in the Drosophila compound eye appears to be mediated by signals from neighboring cells within each developing cluster of cells in the eye–imaginal disc (for review, see Greenwald and Rubin 1992). A rapidly growing number of genes required in photoreceptor cell determination, particularly that of the R7 cell, have been identified. These include sevenless (sev), which encodes a receptor tyrosine kinase required in the R7 cell, and boss, the ligand for the Sev protein that is required specifically in the R8 cell (Hafen et al. 1987; Reinke and Zipursky 1988; Simon et al. 1989; Kramer et al. 1991). The recent isolation of mutations in Ras1, and the positive and negative regulators of Ras1, Sos, and Gap1, have demonstrated that R7 cell determination involves activation of Ras1 (Rogge et al. 1991, 1992; Simon et al. 1991, Bonfimi et al. 1992, Buckles et al. 1992; Gaul et al. 1992). Although the direct effector for Ras1 has not been identified, the Drosophila homolog of the mammalian serine/threonine kinase Rafl has been shown to act downstream of Ras1 (Dickson et al. 1992). Considerably less is known about the events that lead to the specification of the other photoreceptor cell fates in the compound eye, although Notch, scabrous, Ellipse, and argos (also referred to as giant lens and strawberry) are examples of other genes that may act in intercellular signaling events in the compound eye (Baker and Rubin 1989; Cagan and Ready 1989, Baker et al. 1990; Mlodzik et al. 1990; Freeman et al. 1992; Kretzschmar et al. 1992; Okano et al. 1992).

Many of the genes important in photoreceptor cell determination are expressed in subsets of cells and for restricted periods during their development. However, the mechanisms regulating the temporal and spatial expression of the genes important in eye development are not understood. Several transcription factors have been identified that are required in the developing compound eye—rough, glass, seven-up, sina, and yan (for review, see Moses 1991; Lai and Rubin 1992). The seven-up gene is a member of the steroid hormone receptor superfamily, although the ligand for this putative transcription factor is unknown (Mlodzik et al. 1990). Mutations in seven-up result in the transformation of four outer photoreceptor cells toward an R7 cell fate. The sina locus is essential for R7 cell development and encodes a putative transcription factor with a potential metal-binding domain (Carthew and Rubin 1990). The rough gene encodes a protein with a homeo domain required for the development of photoreceptor cells R3 and R4 (Tomlinson et
al. 1988). Mutations in glass specifically remove photoreceptor cells, leaving the other cells in the ommatidia intact. The glass gene encodes a protein with five zinc fingers of the Cys-His class and has been shown to be a DNA-binding protein [Moses et al. 1989; Moses and Rubin 1991]. Recently, a putative negative regulator of photoreceptor cell development, referred to as yan, has been identified [Lai and Rubin 1992]. Loss-of-function mutations in yan result in the appearance of supernumerary R7 cells.

To identify additional genes important for the development or physiology of the compound eye, we screened P-element insertion lines for eye defects. In this paper we describe the identification of a mutation in the tramtrack (ttk) gene, a proposed negative regulator of transcription. The 69-kD Tramtrack protein (Ttk) was purified previously from embryo extracts on the basis of binding the regulatory regions of two pair-rule genes, fushi tarazu (ftz) and even-skipped (eve). The ttk gene was subsequently cloned and shown to encode two proteins, 69 and 88 kD (p69 and p88), with different pairs of zinc fingers [Harrison and Travers 1990; Brown et al. 1991a; Read and Manley 1992]. It has been proposed that p69 may play a role in the repression of zygotic gene expression of ftz as well as other genes during the early nuclear division cycles prior to the cellular blastoderm stage [Brown et al. 1991a]. This suggestion is supported by recent experiments indicating that p69 can act to repress expression of several pair-rule genes in embryos, including ftz and eve. Misexpression of high levels of p69 in transgenic embryos, under the control of a heat shock promoter, significantly reduces the striped pattern of expression typical of ftz and eve [Read et al. 1992; Brown and Wu 1993]. p88 has been shown to bind to a regulatory region of the segment polarity gene engrailed (en); however, ectopic expression of p88 does not appear to have any effect in embryos [Read and Manley 1992]. These experiments raise the intriguing question as to whether either p69 or p88 is normally a transcriptional repressor of segmentation genes when expressed in the normal distribution of cells at wild-type levels.

In this study we report that in the P-element insertion allele ttk1, there was a defect in cell fate determination in the eye resulting in the generation of an increase in the average number of R7 cells and a decrease in the average number of outer photoreceptor cells per ommatidium. The mRNA encoding p88 was missing in ttk1, indicating that the phenotype was attributable to the absence of p88. The role of p88 may be to repress inappropriate expression of genes which, if expressed, would result in disruption of the normal pattern of eye development. Consistent with the proposal that p88 is a transcriptional repressor, we detected increased levels of en expression in several ttk1 larval tissues; the most dramatic effect was detected in the salivary glands and fat bodies, and a smaller effect was observed in eye–imaginal discs. Null ttk alleles displayed early embryonic lethality and, in mosaic patches in the adult eye, transformation of ommatidial cell types into nonommatidial tissue. Thus, p69 appeared to be important for embryonic development, and both p69 and p88 were required in the eye.

Results

Identification of a mutation in the ttk locus

We identified a P-element-induced mutation in the ttk locus, ttk1, in a screen for recessive autosomal mutations affecting the development of the adult compound eye. A collection of homozygous viable P-element insertion lines [Karpen and Spradling 1992] were screened by examining the deep pseudopupil for mutations that caused defects in the arrangement or number of photoreceptor cells. The deep pseudopupil is an optical image superimposed on the surface of the eye appearing as seven large circles [Franceshini and Kirschfeld 1971]. Mutations that disrupt the number or arrangement of photoreceptor cells similarly perturb the deep pseudopupil.

Evidence that the mutation identified was in ttk was based on several criteria. First, on the basis of in situ hybridization to polytene chromosomes and Southern blot analyses, the mutant flies contained a single P-element insertion in the same third chromosome position, 100D, as the ttk gene [data not shown]. Second, the P element interrupted the ttk-transcribed region. The ttk locus is expressed as two major mRNAs, 4.2 and 5.0 kb, encoding proteins of 643 and 813 amino acids with predicted molecular masses of 69 and 88 kD [referred to as p69 and p88]. p69 and p88 are identical through their first 286 amino-terminal residues but contain unique carboxy-terminal ends with completely different pairs of zinc fingers [Fig. 1B]. On the basis of Southern blot analyses, we found that the P element inserted into an intron of a 4.2-kb mRNA and an exon of a 5.0-kb mRNA [Fig. 1A].

Evidence that the mRNAs interrupted by the P element encoded the Ttk proteins was obtained by isolating cDNAs of 3.7 and 4.5 kb, corresponding to the 4.2- and 5.0-kb mRNAs from a third-instar larvae eye disc library and comparing the sequences to the deduced amino acid sequences of the previously reported ttk cDNAs [Harrison and Travers 1990; Brown et al. 1991a; Read and Manley 1992]. The only major deviations between the ttk sequences obtained in the current report and the previously reported ttk cDNA sequences were in the 5′- and 3′-untranslated regions. The first 192 nucleotides in the 5′-untranslated region were unrelated to the sequence at the 5′ end of the previously reported cDNAs. In addition, the length of the 3′-untranslated region in the 4.5-kb eye disc cDNA was considerably longer than the 3′-translated region of the previously isolated cDNA encoding p69 [Fig. 1B]. Two amino acid differences between the current and previously analyzed p88 sequences, probably owing to sequence polymorphisms, occurred in the unique carboxy-terminal region [see Fig. 1B]. The heterogeneity in the 5′- and 3′-untranslated sequences could reflect differences in maternal and zygotic mRNAs because the previous cDNAs were isolated from libraries.

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**Figure 1.** Physical map of ttk cDNAs and genomic region in wild-type, ttk\(^{1}\), and ttk\(^{1\epsilon 11}\). (A) Structure of ttk genomic DNA in wild-type, ttk\(^{1}\), and ttk\(^{1\epsilon 11}\). A 16.0-kb portion of the ttk\(^{2}\) genomic region, inserted in ttk-1, is represented by the horizontal line demarcated in kilobase pairs and by the restriction sites BamHl (B), EcoRl (E), and SalI (S). The insertion site of the mutator P-element is indicated by the vertical arrow above coordinate 7.5. This insertion site corresponds to nucleotide 2741 in the 4.5-kb cDNA, 3' to the coding region, that ends at nucleotide 2285. The P-element inserted such that its 3' end is proximal to the 5' end (coordinate 0) of the genomic region. The physical map of ttk\(^{1\epsilon 11}\) is shown above the map of ttk\(^{1}\). Approximately 1.7 kb was deleted from the 3' end of the P-element and 0.8 kb from the flanking genomic DNA. The approximate region transcribed in the vicinity of the P-element insertion site, determined by probing Southern blots with a\(^{32}\)P-labeled cDNA prepared from adult head RNA, is indicated by the solid bar below the ttk\(^{1}\) physical map. The approximate genomic regions encoding the 3.7- and 4.5-kb cDNAs, corresponding to the 4.2- and 5.0-kb mRNAs, are indicated. The hatched area represent the region encoding the amino-terminal 286 amino acids common to both cDNAs. The regions encoding the 355 and 525 amino acids unique to the 3.7- and 4.5-kb cDNAs are indicated by the light and dark stippled boxes, respectively. The 5'- and 3'-untranslated regions are represented by the open bars. (B) Structures of the ttk cDNAs. The two top horizontal lines represent the 3.7- and 4.5-kb cDNAs encoding p88 and p69, respectively. The thick portions of the lines represent the coding regions. The bottom line demarcates the amino acid and nucleotide positions encoded in the cDNAs. The amino acids are represented by the numbers above the lines and the nucleotides by the numbers below the lines. The common 5'-untranslated region and amino-terminal 286 amino acids encoded by the 3.7- and 4.5-kb cDNAs are indicated by hatching. The coding regions unique to the 3.7- and 4.5-kb cDNA are represented by the light and dark stippled regions, respectively. The positions of the distinct pairs of zinc fingers in p88 and p69 are shown. Several differences are shown between the 3.7- and 4.5-kb cDNAs isolated from the eye disc library and the cDNAs isolated previously from embryonic libraries. The 192-nucleotide region at the 5' end of the untranslated region, indicated by the thick black line, was completely divergent. The length of the 3'-untranslated region in the cDNAs encoding p69 also diverged significantly. The positions of the 3' ends of the cDNAs isolated from the eye disc and embryonic libraries are shown. M and K above the 3.7- and 4.5-kb cDNAs represent the two additional amino acids, methionine and lysine, at the amino terminus of the eye disc cDNAs. Also noted is the arginine (R), instead of proline (P), at residue 358 and the glutamine (Q), instead of glutamic acid (E), at residue 524. pttt\(^{37}\), but not pttt\(^{45}\), contained a poly(A) tail.

Prepared from either early embryos or mixed staged embryos. The cDNAs isolated in the current analyses were obtained from an eye disc cDNA library. Alternatively, the unrelated 5'- and 3'-untranslated regions could be the result of different RNA splicing patterns in embryos and larval eye-imaginal discs.

A third piece of evidence that the mutation was in ttk was obtained by probing an RNA blot containing wild-type and ttk RNA. Using isoform-specific probes, we found that the wild-type 4.2-kb mRNA was missing and the 5.0-kb mRNA was truncated to 4.2 kb [Fig. 2]. Thus, the concentration or size of both ttk mRNAs was affected in the P-element insertion line.

Fourth, the mutant phenotype was restored to wild type in a high percentage of lines in which the P element was mobilized from the third chromosomal position at...
1.7 kb was deleted from the 3' end of the P element and 0.8 kb of flanking genomic DNA (Fig. 1). The 0.8-kb deletion removed the region encoding the zinc fingers in p69. Even if the truncated p69 protein was stably expressed, it was likely to be devoid of any DNA-binding capacity. Therefore, ttk<sup>eii</sup> affected both Ttk proteins.

**Figure 2.** Northern blot analyses of ttk RNA expressed in ttk<sup>1</sup>. RNAs prepared from wild-type (lanes 1,3,5) and ttk<sup>1</sup> (lanes 2,4,6) adult flies were fractionated on a 1% agarose, 6% formaldehyde gel, transferred to a nylon membrane, and probed with either the entire 3.7-kb cDNA (lanes 1,2) or the unique regions of the 3.7-kb (lanes 3,4) and 4.5-kb cDNAs (lanes 5,6). The 3.7- and 4.5-kb cDNAs contain the entire coding regions of the 4.2- and 5.0-kb mRNAs, respectively. The probe specific to the 3.7-kb cDNA was a 0.8-kb SacI–EcoRI fragment from the extreme 3' end of the cDNA. The probe specific to the 4.5-kb cDNA was a cDNA that was a truncated version of the 4.5-kb cDNA extending 2.6 kb from the 3' end of the 4.5-kb cDNA. The 5.0- and 4.2-kb mRNAs are indicated.

**Expression of ttk RNA during development**

Expression of ttk RNA during Drosophila development was investigated by probing an RNA blot, containing RNA prepared from various stages throughout development, with ttk DNA (Fig. 3). The DNA probe was to the region common between the 4.2- and 5.0-kb mRNAs to enable the relative concentrations of the two predominant mRNA forms to be assessed. The results of the analyses demonstrated that ttk was expressed at the highest levels in embryos and in the adult. Longer exposures of the autoradiograph demonstrated that ttk is expressed at lower levels throughout development. The 5.0-kb mRNA was expressed at levels slightly higher than those of the 4.2-kb mRNA during development, whereas both ttk mRNAs were expressed in approximately equal proportion in the adult. In addition to the 5.0- and 4.2-kb mRNAs, a slightly less abundant mRNA, ~3.6 kb, was observed specifically in the adult (Figs. 2 and 3), however, the structure of this mRNA has not been determined (see Materials and methods). Of 19 excision lines, 14 reverted to wild type, demonstrating that the ttk<sup>1</sup> phenotype was induced by the P-element rather than by a spontaneous mutation. One excision line exhibited a phenotype similar to ttk<sup>1</sup>, and the remaining four lines were homozygous lethal. One of the lethal excision alleles, ttk<sup>eii</sup>, appeared to be a null allele (see below).

**Northern, Southern, and DNA sequencing analyses suggested that p88 was eliminated in ttk<sup>1</sup> and both p69 and p88 were affected in ttk<sup>eii</sup>.**

The site of the P-element insertion in ttk<sup>1</sup> was determined by DNA sequence analysis (see Materials and methods). We found that the P element inserted 0.45 kb from the 3' end of the coding region of the 4.5-kb cDNA. Therefore, the truncated 5.0-kb mRNA expressed in ttk<sup>1</sup> could still potentially encode p69. Because the truncated 5.0-kb mRNA in ttk<sup>1</sup> was expressed at a level similar to that of the full length 5.0-kb mRNA in wild type (Fig. 2), it is likely that expression of p69 was unaffected in ttk<sup>1</sup>. The p88 protein was eliminated or dramatically reduced in ttk<sup>1</sup> because the 4.2-kb mRNA was not detected in RNA blots (Fig. 2). Insertion of the P element in the intron of the 4.2-kb mRNA may have interfered with the processing of this mRNA.

To determine whether ttk<sup>eii</sup> was associated with a deletion, Southern blots containing ttk<sup>eii</sup> fly genomic DNA were probed with a series of small DNA probes from the 4.5-kb cDNA [data not shown]. We found that ~1.7 kb was deleted from the 3' end of the P element and 0.8 kb of flanking genomic DNA (Fig. 1). The 0.8-kb deletion removed the region encoding the zinc fingers in p69. Even if the truncated p69 protein was stably expressed, it was likely to be devoid of any DNA-binding capacity. Therefore, ttk<sup>eii</sup> affected both Ttk proteins.
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been determined. The relatively faint band, ~2.1 kb [shown in Fig. 2, lane 1], was not observed consistently and might be a degradation product.

**Fewer outer photoreceptor cells and extra R7 cells in many ttk^1 ommatidia**

A wild-type compound eye contains ~800 facets or ommatidia, each of which contains eight photoreceptor (R) cells. Six of the photoreceptor cells, R1–R6, extend the full depth of the ommatidia and contain large rhabdomeres positioned along the periphery of the ommatidia. The rhabdomeres of the R7 and R8 cells are smaller and occupy the central upper (distal) and lower (proximal) portions of the ommatidia, respectively. Consequently, only seven of the eight photoreceptor cells are present in any given cross section.

The ttk^1 retina was found to contain >50% aberrant ommatidia with abnormal numbers and positioning of photoreceptor cells. Shown in Figure 4 are cross sections of the distal region (R7 level) of the compound eye. In contrast to wild-type ommatidia, which always contain seven R cells [Fig. 4A], ttk ommatidia were characterized by a variable number of photoreceptor cells [Fig. 4B]. Table 1 lists the number of outer (R1–R6) photoreceptor cells and R7 cells in each ommatidium. The identities of the photoreceptor cells, as outer or R7 cells, were based on the position and size of the rhabdomeres and on genetic criteria [see below]. Among ommatidia with altered numbers of R1–R6 cells, 98% displayed a decreased number of outer photoreceptor cells. However, among ommatidia with aberrant numbers of R7 cells, 87% had extra R7 cells. Typical ommatidia with decreased numbers of outer photoreceptor cells and additional R7 cells are shown in Figure 4C, D. The average numbers of R1–R6 and R7 cells were 5.4 and 1.4 per ommatidium, respectively; however, the exact number of cells was difficult to score as some rhabdomeres were fused or split. If only abnormal ommatidia were considered, the average number of outer R cells dropped further to 4.9 and R7 cells increased to 1.8. The extra R7 cells did not appear to result from the simple transformation of R1–R6 cells into R7 cells, as there were many ttk^1 ommatidia with extra R7 cells in addition to the normal complement of six outer photoreceptor cells [Table 1]. Other ttk^1 ommatidia contained one R7 cell but fewer than six outer photoreceptor cells [Table 1]. Examination of the proximal cross section (R8 level) demonstrated that all of the ommatidia in ttk^1 contained one small rhabdomere, indicating that there was no defect in the number of R8 cells [data not shown]. In addition to the defects in the eyes, the fertility of ttk^1 flies was reduced significantly, indicating that ttk^1 may also have a role in oogenesis.

To obtain additional evidence that the extra cells with small rhabdomeres are R7 cells, a double mutation was constructed between ttk^1 and a null allele of the *ninaE* locus, *ninaE^117*, which disrupts the opsin gene expressed in the R1–R6 cells. The *ninaE^117* mutation results in degeneration of the outer photoreceptor cells prior to eclosion, therefore, only a single central small rhabdomere corresponding to the R7 cell appears in distal cross sections [Fig. 4E; O'Tousa et al. 1989]. Occasionally, the central R7 rhabdomere in *ninaE^117* was split; however, no ommatidium contains more than one small rhabdomere [Fig. 4E]. In contrast to *ninaE^117*, many ttk^1, *ninaE^117*, ttk^1 double mutant ommatidia contain more than one rhabdomere, demonstrating that the extra R cells were refractory to the R1–R6 specific degeneration caused by the *ninaE^117* mutation [Fig. 4F]. Hence, it appears that the additional small rhabdomere-containing cells were R7 cells.

Examination of developing eye–imaginal disc with a neuronal-specific antibody indicated that the stereotypic pattern of R-cell development was disrupted in ttk^1 [data not shown]. Differentiation of the photoreceptor cells begins in the third-instar larval eye–imaginal disc in a groove referred to as the morphogenetic furrow. Anterior to the furrow, cells are undifferentiated, and posterior to
Null mutants for ttk result in embryonic lethality and, in mosaic flies, transformation of ommatidial to nonommatidial tissue

ttk<sup>1</sup> did not appear to be a null allele because 4 of the 19 lines in which the P element was mobilized from the third chromosomal position at 100D were homozygous lethal. Evidence that the lethality associated with the lethal excision lines was not the result of insertion of the mobilized P element in other essential genes at new chromosomal positions, or to a background lethal mutation, was obtained by placing ttk<sup>1</sup> and the lethal excision alleles in °trans with a deletion of the corresponding region, Df(3R)awd<sup>KRB</sup>, resulting in lethality. The strongest ttk excision allele, ttk<sup>le11</sup>, may be a null allele, as ttk<sup>le11</sup> in °trans with Df(3R)awd<sup>KRB</sup> was similar in phenotype to ttk<sup>le11</sup> homozygotes. The lethality associated with ttk<sup>le11</sup> homozygotes appeared to result from an arrest in development early in embryogenesis, before dorsal closure (Fig. 5). The ttk<sup>le11</sup> embryos formed segments but displayed severe perturbations of cuticular structures. In addition, the Filzkörper and head

The effect of the strong ttk allele, ttk<sup>le11</sup>, on formation of the adult eye was examined by performing somatic mosaic analysis. Homozygous ttk<sup>le11</sup> mutant cells were genetically marked using an ectopic copy of the white (w<sup>+)</sup> gene [a recessive gene affecting eye color], inserted on the third chromosome at 90F, as a cell autonomous marker. Clones of homozygous mutant cells were made by inducing mitotic recombination with X-ray irradiation in the third larval instar. Photoreceptor cells that were w<sup>-</sup> were homozygous mutant in an otherwise wild-type eye. Ommatidia in small mutant clones appeared to be devoid of any photoreceptor cells or cone cells [Fig. 6]. Because there is no lineage restriction in the formation of an ommatidium, cells of the different genotypes mix at the borders of mutant clones. By examination of phenotypically wild-type photoreceptor cells in the border areas for the white marker, it is possible to assess which cells require the ttk gene product. We found that all photoreceptor cells were genotypically wild type, indicating that the ttk gene product was required cell autonomously in each of the photoreceptor cells.

Occasionally, compound eyes with large mutant clones were obtained, resulting in a strong rough eye phenotype in the region surrounding the mosaic patch [Fig. 7]. The rough areas were characterized by the presence of extra mechanosensory bristles between some

![Figure 5. Cuticle preparation of wild-type and ttk<sup>le11</sup> embryos.](genesdev.cshlp.org)
ommatidia and the absence of mechanosensory bristles between others. The size and shape of many of the facets was also altered. No w- areas were observed in the rough patches, apparently owing to complete loss of ommatidia in the mutant regions. Areas that would normally consist of ommatidial tissue in a wild-type eye were transformed to nonommatidial tissue with numerous hairs. These results indicated that the null mutation in ttk prevented development of all normal cell types in the compound eye.

Enhancement of en expression in several ttk1 larval tissues

The p69 protein has been proposed to be a transcriptional repressor of several pair-rule genes, including ftz, eve, and hairy, and p88 has been suggested to be a repressor of the segment polarity gene en [Harrison and Travers 1990; Brown et al. 1991a; Read and Manley 1992; Read et al. 1992; Brown and Wu 1993]. To provide in vivo evidence that p88 is a repressor, we compared ftz, hairy, and en expression in wild-type and ttk1 larval tissues by immunostaining. The intensity of staining with ftz and hairy was indistinguishable between wild-type and ttk1. However, several tissues expressed en at a higher level in ttk1 than in wild type. The most dramatic difference was observed in the salivary glands and fat bodies (Fig. 8A,B). A much smaller, but reproducible, enhancement of expression was also detected in ttk1 eye-imaginal discs (Fig. 8C,D), where expression was detected in all of the photoreceptor cells, the cone cells, and undetermined cells behind the morphogenetic furrow. Although the increase in en expression in eye discs was not dramatic, in a blind experiment, 20 eye discs were all successfully identified as ttk1 or wild type on the basis of en staining. In other larval tissues, such as the brain, central nervous system, and wing, leg, and haltere discs, the intensity of en staining was similar in ttk1 and wild type. The en embryonic expression pattern of 14 major stripes typical

of wild-type embryos was also detected in nearly all ttk1 embryos [data not shown]. However, in a few ttk1 embryos (<2%), the stripes were broader than usual [data not shown]. Additional experiments will be required to determine whether this was the result of a background mutation or a slight effect on en expression in occasional ttk1 embryos. To determine whether there was ectopic expression of ftz, eve, and en in ttk1 embryos were collected from a ttk1+/+ inter se cross and stained with antisera. No ectopic staining was detected with ftz, eve or en.

Discussion

In this paper we describe the identification and characterization of a mutation in ttk, a locus required for the development of the adult compound eye. The original ttk allele, [ttk1], was isolated in a genetic screen, using
Figure 8. Expression of en in ttk1 larval tissues. Wild-type and ttk1 tissues from third-instar larvae stained with an en mouse monoclonal antiserum are shown. (A) Wild-type salivary gland (S) and fat body (F); (B) ttk1 salivary gland (S) and fat body (F); (C) wild-type eye (E)-antennal (A) imaginal disc; (D) ttk1 eye-antennal imaginal disc.

P-element insertion mutagenesis, to identify loci required for the development or physiology of the adult compound eye. The 69-kD ttk protein was purified previously on the basis of binding the transcriptional control regions of two pair-rule genes ftz and eve (Harrison and Travers 1990; Brown et al. 1991a; Read and Manley 1992). The ttk gene was subsequently cloned by screening expression libraries with the ttk DNA-binding site and shown to encode two proteins, p69 and p88, with different pairs of zinc fingers of the Cys-His class (Harrison and Travers 1990; Brown et al. 1991a; Read and Manley 1992). We have shown that in the ttk1 allele, there is a defect in the development of the compound eye characterized by an increase in the average number of R7 cells and a decrease in the average number of R1–R6 cells. In contrast to ttk1, the null allele ttk1null was characterized by early developmental arrest and embryonic lethality. These embryos were segmented but displayed severe defects in cuticle formation. Somatic mosaic analyses demonstrated that the null mutation in ttk results in transformation of normal cell types in the compound eye with nonommatidial tissue.

Possible source of the extra R7 cells in ttk1

In addition to ttk1, several other mutations have been identified that result in supernumerary R7 cells. One example is the seven-up mutation (Mlodzik et al. 1990). Absence of seven-up function results in the transformation of R1, R3, R4, and R6 cells to an R7 cell fate. Additional photoreceptor cells with small rhabdomeres, presumably R7 cells, are observed in many ommatidia in distal eye sections from rough1 flies (Tomlinson et al. 1988). The rough and seven-up gene products may act to repress development of the R7 cell fate in other photoreceptor cells. Consistent with this proposal, ectopic expression of rough in the R7 cell precursor results in transformation of the R7 precursor into an R1–R6 type of photoreceptor cell (Baster et al. 1990; Kimmel et al. 1990). The yan locus, encoding an ETS DNA-binding domain, is another putative negative regulator of R7 cell development (Lai and Rubin 1992).

Non-neuronal cone cell precursors and mystery cells can also be converted into an R7 cell fate. The extra R7 cells in yan appear to be derived from cone cell precursors or from the general pool of uncommitted cells. Expression of a constitutively active Sevenless protein appears to cause the conversion of mystery cells and cone cell precursors into R7 cells (Basler et al. 1991). Similarly, the formation of supernumerary R7 cells from cone cell precursors and mystery cells is induced by the expression of a dominant activating Ras1 protein, loss-of-function Gap1 alleles, or ectopic expression of boss (Fortini et al. 1992).

The source of the extra R7 cells in the ttk1 allele may be the cone cell precursors and/or mystery cells because nearly 60% of ommatidia with supernumerary R7 cells contained too many outer photoreceptor cells to account for the additional R7 cells. Most notably, >40% of the ommatidia with extra R7 cells displayed the normal complement of six outer photoreceptor cells. Therefore, it is unlikely that these extra R7 cells developed from R1–R6 cell precursors. However, it cannot be excluded that some of the additional R7 cells in ttk1 developed from R1–R6 cell precursors.

p69 and p88 appear to have different roles

The ttk gene is expressed as two alternatively spliced mRNAs encoding two proteins, p69 and p88, which share common amino termini but differ from each other over the carboxy-terminal 55% and 65% of the proteins, respectively. Both p69 and p88 contain different pairs of zinc fingers, suggesting that each Ttk protein may regulate different target genes. Consistent with this proposal, p69 and p88 have been shown recently to have distinct
DNA-binding specificities in vitro (Read and Manley 1992). The p69 protein has been shown to bind in vitro to multiple sites within important regulatory elements of the pair-rule genes ftz and eve, and p88 binds the regulatory element of the segment polarity gene en (Harrison and Travers 1990; Brown et al. 1991a; Read and Manley 1992). The differences in DNA-binding specificities of p69 and p88 in vitro raises the issue as to whether the proteins have different roles in vivo.

The analyses described in this report indicate that p69 and p88 have different roles. The ttk mutant, in which the mRNA encoding p88 was not detected, was characterized by extra R7 cells and a decreased number of outer photoreceptor cells. In contrast to ttk, the ttk excision allele, which affects both p69 and p88, displayed early embryonic lethality and in mosaic flies, transformation of all ommatidial cells into nonommatidial tissue containing numerous hairs. This phenotypic analysis indicates that p69 is most important during embryogenesis and both p69 and p88 are required in the compound eye. The different roles of the two Ttk proteins do not appear to be the result of different expression patterns in the eye–imaginal disc. Using isofor-specific antisera, it appears that both p69 and p88 are expressed predominantly in the subretinal cells and cone cells (W.-C. Xiong and C. Montell, unpubl.). These results are consistent with the proposal that the two different Ttk proteins have different target genes in vivo.

The replacement of ommatidial tissue with nonommatidial tissue suggests that there is an alteration in cell fate determination in the ttk mosaic patches. If cell death was responsible for the absence of ommatidial units, then ttk mosaic eyes would be expected to be characterized by decreased numbers of ommatidia without the substitution of nonommatidial tissue. We propose that the role of p69 is to repress expression of genes that are incompatible with development of all normal cell types in the compound eye.

Our results suggesting that p69 is more important than p88 during embryogenesis are supported by recent experiments examining the effects of ectopic expression of high levels of p69 and p88 in an otherwise wild-type embryo (Read et al. 1992; Brown and Wu 1993). Misexpression of p69 results in repression of several pair-rule genes and causes embryonic lethality characterized by severe cuticular defects. Conversely, ectopic expression of p88 had no apparent effect on gene expression in the embryos or on cuticle formation, suggesting that p88 does not appear to affect segmentation gene expression in the embryo. Perhaps p88 functions to repress expression of segmentation genes only in concert with other factors that are not expressed in the embryo but are expressed later in development. Despite these observations, it seems likely that p88 has some function during embryogenesis, as the ttk mutant was lethal in trans with a deficiency for the ttk locus.

Although recent studies indicate that p69 represses expression of several pair-rule genes in embryos, the typical pair-rule phenotype, characterized by deletion of alternating segments, was not observed as a result of the disruption of both Ttk proteins in ttk embryos. The gross effect on cuticle formation in ttk embryos and in embryos ectopically expressing p69 suggests that the Ttk proteins may have some targets in embryos that are not segmentation genes. Our results indicate that neither ftz, eve, nor en was misexpressed in ttk embryos; however, maternal Ttk was still present in these embryos. If p69 has a role in repressing other genes, in addition to the segmentation genes, in early embryos, then elimination of maternal Ttk might cause general defects rather than specific segmentation defects. Further studies, including removing maternal Ttk by constructing germ-line mosaics will be required to determine which genes are important in vivo targets for Ttk in the embryo.

Potential role and targets for p88 in the compound eye

The Ttk proteins have been shown to display divergent DNA-binding specificities and have been suggested to play roles as transcriptional repressors of segmentation genes such as ftz, eve, and en (Read and Manley 1992). Therefore, the mechanism by which p88 could regulate gene expression in the eye might be transcriptional repression. Consistent with the proposal that p88 is a transcriptional repressor, we have shown that expression of en is enhanced dramatically in the larval salivary glands and fat bodies and increased to a lower extent in eye–imaginal discs. We cannot conclude that the disruption in normal eye development in ttk embryos was specifically the result of misexpression of en; however, it is likely that p88 acts directly as a transcriptional repressor of en because p88 has been shown previously to bind the promoter region of en in vitro (Read and Manley 1992).

Because p88 appears to be a transcriptional repressor in vivo, potential targets other than en are loci in which ectopic expression of the wild-type protein partially or fully phenocopy ttk. It is difficult to suggest potential targets for p69 in the compound eye because the phenotype of ttk was unique and was the result of disruption of both Ttk proteins. However, other mutants have been described that display phenotypes reminiscent of ttk. One of the roles of p88 could be to repress expression of genes which, if expressed, would transform non-R7 cell precursors to R7 cells. On the basis of this criterion, one candidate target for p88, boss, encodes the ligand for Sev. Ectopic expression of boss, under control of the heat shock promoter, results in supernumerary R7 cells (Van Vactor et al. 1991). However, if expression of boss were completely derepressed in ttk embryos, one would expect extra R7 cells in all ttk ommatidia. Furthermore, boss could not be the only target for p88, as ectopic expression of boss does not result in fewer outer photoreceptor cells in any ommatidia. Another potential target for p88 is one of the effectors for Ras1.

An alternative role of p88 may be to repress the expression of genes that are normally expressed in the eye, but if misexpressed would result in a mutant phenotype. An example of such a gene is the pair-rule gene hairy. It has been shown recently by somatic mosaic
analysis that hairy is not required for the development of the compound eye [Brown et al. 1991b]. However, ectopic expression of hairy causes a wide range of abnormalities in the compound eye [Brown et al. 1991b]. Because in vitro experiments demonstrate that p69 and not p88 binds the hairy promoter efficiently, the observation that the ttk \(^1\) mutation had no effect on hairy expression in larval eye–imaginal discs was not surprising. Analysis of hairy expression in ttk\(^{tet1}\) mosaic patches in eye discs would be required to determine whether p88 regulates hairy expression during eye development. Future experiments directed at examining the consequences of over-expressing en in eye–imaginal discs, salivary glands, and fat bodies might provide evidence in support of a physiologically relevant role for p88 in repressing en expression.

**Materials and methods**

**Genetics**

The ttk mutant (ttk\(^{-}\)) was identified among 6000 homozygous viable P-element insertion lines generated in Dr. Allan Spradling’s laboratory [Department of Embryology, Carnegie Institution of Washington, Baltimore, MD]. These fly stocks typically contained one, and occasionally two, autosomal copies of a modified mutator P-element consisting of a rosy \(^{+}\) [ry\(^{+}\)] gene, a copy of the pHSS7 plasmid, and a β-galactosidase reporter gene.

The ttk\(^1\) allele was identified by viewing the image of the deep pseudopupil as described previously [Franceschini and Kirschfeld 1971a]. Briefly, w\(^{+}\) flies were anesthetized with ether, transferred to a clear plexiglass platform, and examined with a Zeiss stereomicroscope by directing intense white light from below the flies using a biforcated fiber optic cable. The optical neutralization technique [Franceschini and Kirschfeld 1971] was used as a secondary screen by introducing a drop of immersion oil into a ring of dried nail polish on a microscope slide and immersing dissected fly heads in the immersion oil. A coverslip was placed on top of the sample, and the image of the rhabdomeres was examined on a Nikon Microphot-FXA microscope using very bright anodic release illumination with the condenser diaphragm iris closed to the minimum opening.

To demonstrate that the P-element induced the ttk\(^1\) mutation, the P-element was mobilized by crossing ttk\(^1\) flies with a stock referred to as jumpstarter, cn; D2-3, ry\(^{+}\), Sb/TM6, which provides the P-element transposase required in trans for position. Among 19 ry\(^{+}\) lines that were established, 14 reverted to wild type, 4 were recessive lethals, and 1 homozygous viable line exhibited a mutant eye phenotype.

To confirm that ttk\(^1\) mapped to 100D near the right teleomere of the third chromosome and to ascertain whether ttk\(^1\) was a null allele, we crossed ttk\(^1\) to a stock, Df(3R)M\(^{kk8}\), containing a small deficiency at 100D [Biggs et al. 1990].

Somatic mosaic analysis was performed by generating X-ray-induced mitotic recombination between ttk\(^{+}\) and ttk\(^{-}\) chromosomes. To differentiate ttk\(^{+}\) and ttk\(^{-}\) clones, the white gene was used as a cell autonomous marker. Heterozygous, w\(^{+}\); ttk\(^{tet1}\)/w, P[w\(^{+}\)] ttk\(^{-}\), late first-instar larvae at 48 ÷ 6 hr of development were exposed to 250 rads for 5 min to induce mitotic recombination. The w\(^{118}\), P[w\(^{+}\)] D3 stock has a P-element containing a w\(^{+}\) gene proximal to the ttk gene at position 90F on the third chromosome. Mosaic eyes were examined by scanning and transmission electron microscopy [TEM] as described below.

The ninaE\(^{H}\), ttk\(^1\) double mutant was created by meiotic recombination.

**Electron microscopy**

The ttk eye phenotype was examined at the ultrastructural level by TEM. Hemisected fly heads were fixed in paraformaldehyde and glutaraldehyde, postfixed in osmium tetroxide, dehydrated in an ethanol series, infiltrated in a mixture of propylene oxide and Spurr’s medium, and imbedded in Spurr’s medium as described [Porter et al. 1992]. Thin sections were examined by TEM. Scanning electron microscopy (SEM) was performed by placing dissected adult fly heads on aluminum pin mounts, using double-sided transparent tape, and viewed on an Amray SEM model 1810. The fly heads were not sputter-coated with platinum or treated in any way before SEM analyses.

**Isolation of ttk genomic DNA and determination of P-element insertion site**

The DNA sequences flanking the P-element insertion site were recovered by the plasmid rescue technique. In this technique, the pHSS7 plasmid inserted into the P-element is used to facilitate cloning the genomic DNA flanking the 5’ end of the P-element. To perform the plasmid rescue technique, ttk genomic DNA was digested with XbaI and SpeI, a portion of the DNA, roughly equal to two fly equivalents of DNA, was then ligated under conditions to favor intramolecular ligation and transformed into competent Escherichia coli prepared by the procedure of Hanahan [1983]. A 4.5-kb segment of genomic DNA, extending to a SpeI site, was recovered in the rescued plasmid pRP ttk and used to screen a wild-type bacteriophage λ genomic library. Among the genomic clones isolated was ttk\(^{-}\), which contained a 16.0-kb insert. The P-element insertion site in ttk\(^{-}\) was determined by performing double-stranded DNA sequence analysis using pRP ttk and a primer, GTATACTTCGG-TAACGTTCGGCTTTC, complementary to the 5’ end of the P-element [nucleotides 56–32]. The extent of genomic and P-element DNA deleted in ttk\(^{tet1}\) was determined by Southern blot analyses of genomic DNA prepared from each fly strain.

**RNA blots and identification of transcribed region**

To identify the transcribed region, ttk\(^{-}\) was digested with various restriction enzymes, transferred to GeneScreen Plus [New England Nuclear], and probed with \(^{32}\)P-labeled cDNA prepared from adult RNA. Restriction fragments, extending from the SalI site at coordinate 3.1 to the BamHI site at coordinate 11.5, hybridized most strongly with the cDNA [Fig. 1]. To perform RNA blots, total RNA was prepared as described, transferred to GeneScreen Plus, and probed with the \(^{32}\)P-labeled DNA fragments indicated in the legends to Figures 2 and 3.

**Isolation and sequencing of cDNAs**

An eye disc cDNA library was screened with the \(^{32}\)P-labeled 4.5-kb genomic region contained in the rescued plasmid pRP ttk. The filters were hybridized and washed as described [Montell et al. 1987]. Among the cDNA isolated was pckt-3.7 [3704 nucleotides] and pckt-4.5 [4524 nucleotides], which corresponded to the 4.2- and 5.0-kb mRNAs. pckt-3.7, but not pckt-4.5, contained a poly(A) tail. To map the approximate genomic region encoding each cDNA, a Southern blot containing ttk\(^{-}\) DNA was probed with \(^{32}\)P-labeled pckt-3.7 and pckt-4.5.

The cDNAs were sequenced by the dideoxy chain termination method using Sequenase. The template DNAs used for the
DNA sequencing were generated by the random shearing of pckt-3.7 and pckt-4.5 by sonication and the subcloning of fragments into the SmaI site of M13mp10 as described previously [Montell et al. 1987]. Recombinant M13 clones containing cDNA inserts were identified by plaque hybridizations using 32P-labeled purified pckt-3.7 and pckt-4.5 fragments.

Immunostaining of wild-type and ttk tissues

Wild-type and ttk/+ larval tissues were dissected in 0.1 M sodium phosphate (pH 7.2) and fixed for 45 min in 2% paraformaldehyde, 0.01% sodium iodate, 0.075 M lysine, and 0.037 M sodium phosphate (pH 7.2) as described previously [Tomlinson and Ready 1987] and stained with mouse monoclonal antiserum to ftz and en. A heterogenous overnight collection (18°C) of wild-type and ttk/leu1/+ embryos were fixed and stained with ftz, eve, and en as described previously [Patel et al. 1989]. An overnight collection of ttk/ embryos were also stained with ftz and en as described [Patel et al. 1989].

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Note added in proof

The sequence data described in this paper have been submitted to the EMBL/GenBank data libraries under accession numbers X71626 and X71627.

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