Molecular Characterization of the Drosophila 
*trp* Locus: A Putative Integral Membrane Protein 
Required for Phototransduction

Craig Montell* and Gerald M. Rubin 
Howard Hughes Medical Institute 
and Department of Biochemistry 
University of California 
Berkeley, California 94720

**Summary**

Recent studies suggest that the fly uses the inositol lipid signaling system for visual excitation and that the Drosophila transient receptor potential (*trp*) mutation disrupts this process subsequent to the production of IP$_3$. In this paper, we show that *trp* encodes a novel 1275 amino acid protein with eight putative transmembrane segments. Immunolocalization indicates that the *trp* protein is expressed predominantly in the rhabdometric membranes of the photoreceptor cells.

**Introduction**

The phototransduction cascade in flies appears to share many important features with signal transduction cascades that are initiated by a variety of stimuli such as neurotransmitters, hormones, and growth factors. As in many of these cascades, it appears that the fly’s light-sensitive receptor, rhodopsin, activates a G protein (Blumenfeld et al., 1985; Paulsen and Bentrop, 1986). In flies, the effector for the G protein appears to be phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP$_2$), resulting in the generation of inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (Devarny et al., 1987; Inoue et al., 1988; Bloomquist et al., 1988). Diacylglycerol activates a serine/threonine phosphorylating enzyme referred to as protein kinase C (reviewed by Berridge, 1987); however, it is unclear whether DAG has any role in phototransduction. IP$_3$ has been shown to stimulate the release of Ca$^{2+}$ from internal Ca$^{2+}$ storage vesicles in Limulus ventral photoreceptors (Payne et al., 1986; Payne, 1986) and is presumed to similarly mobilize Ca$^{2+}$ in Drosophila photoreceptor cells. Ca$^{2+}$ in turn regulates a wide variety of cellular processes, including phototransduction (reviewed in Berridge, 1987). By analogy to work in Limulus, the release of Ca$^{2+}$ from the intracellular stores in Drosophila is thought to result in the opening of the light-sensitive ion channels and depolarization of the receptor potential.

Phototransduction does not appear to be a strictly linear cascade, but is controlled by several feedback loops. One example of a feedback loop is the phosphorylation of photoactivated rhodopsin molecules by rhodopsin kinase. This phosphorylation turns off the rhodopsin, thereby preventing it from subsequently activating additional G proteins (transducins). Recent studies indicate that Ca$^{2+}$ also may be important in feedback controls of both vertebrate and invertebrate phototransduction (Koch and Stryer, 1986; Payne et al., 1988).

Many of the genes and proteins important for signal transduction cascades have been identified from a variety of organisms. These include proteins required directly in the cascades, such as receptors (reviewed in Catterall, 1988; Barnard et al., 1987), G proteins (reviewed in Gilman, 1987), and PLC (Stahl et al., 1986; Mayer et al., 1988). Other genes and proteins required indirectly for signal transduction have also been identified. For example, the Ca$^{2+}$-ATPase of the muscle sarcoplasmic reticulum has been cloned (MacLennan et al., 1985; Brandl et al., 1986), and recently the Ca$^{2+}$ release channel protein from rabbit muscle sarcoplasmic reticulum has been purified (Lai et al., 1988). Some of the genes encoding components required directly for phototransduction have been identified in Drosophila. These include four Drosophila opsins (O’Toole et al., 1985; Zucker et al., 1985, 1987; Cowman et al., 1986; Montell et al., 1987; Fryxell and Meyerowitz, 1987) and PLC (Bloomquist et al., 1988).

During the last 20 years, Drosophila mutations that are defective in photoreception and processing of the light-initiated signal in photoreceptor cells have been identified (reviewed in Pak, 1979; Hall, 1982). These mutations provide a genetic approach to the identification and characterization of genes important in signal transduction that have not been identified previously in other organisms. An example of one such mutation is transient receptor potential (*trp*).

The *trp* locus is among the most analyzed of the Drosophila phototransduction mutations. The *trp* mutation was originally identified on the basis of a behavioral phenotype; under bright light conditions, *trp* flies behave as though blind (Cosens and Manning, 1969). The *trp* mutation is also characterized by an electroretinogram phenotype. Electroretinograms measure the change in potential due to extracellular current flow in the eye in response to light. Both wild-type and *trp* flies display a corneal negative electroretinogram in response to light. However, unlike wild-type flies, during continuous bright illumination, the receptor potential in *trp* flies quickly returns to baseline. The response of *trp* flies to a subsequent intense light stimulus is also abnormal. Wild-type flies always display a response to light regardless of the time interval between stimuli; however, *trp* flies require a 60 s dark recovery period after response inactivation and show no response to bright light after 2 s. Under conditions of dim illumination, the *trp* flies are indistinguishable from wild-type flies. Taken together, these data suggest that the *trp* phenotype may arise from depletion during intense illumination of a critical component required for phototransduction.

The *trp* locus appears to encode a protein important in a step intermediate between photoreception and...
the opening of the light-sensitive ion channels (Minke, 1982). The phenotype is not due to a defect in the rhodopsin, as the photopigment properties of trp mutants are normal (Minke, 1982). The light-sensitive channels also appear normal, as only the number but not the shape or size of the quantum bumps are affected in trp (Minke et al., 1975; Minke, 1982). (Bumps are small, discrete depolarization events generated by the absorption of single photons; they sum to produce the receptor potential.)

The nss mutation in the much larger fly, Lucilia, has a phenotype indicative of a detect in a protein very similar to trp (Barash et al., 1988). These studies strongly suggest that the nss gene is the Lucilia homolog of trp. The larger size of the Lucilia eye facilitates pharmacological studies that would be far more difficult in Drosophila. Chemicals, such as IP$_3$, affecting different steps in the phototransduction cascade were used to determine the step disrupted by the nss mutation (Suss et al., 1989). If the site of action of the nss defect is prior to the production of IP$_3$, then introduction of this chemical would be expected to elicit identical responses in the wild type and the nss mutant. However, IP$_3$ was shown to act synergistically with light to accelerate the decline of the nss receptor potential to baseline. These studies indicate that trp, by analogy to nss, has a role subsequent to the production of IP$_3$ (Suss et al., 1989).

In the current paper we describe the molecular characterization of the Drosophila trp gene, which had been identified by rescuing the mutant phenotype (Montell et al., 1985). We show that the 4.1 kb trp RNA encodes a 1275 amino acid protein. The trp protein appears to be a new component required in phototransduction, as it shows no significant similarity to any previously described protein. Analysis of the deduced amino acid sequence suggests that trp contains 8 transmembrane segments. Near the C terminus is a very hydrophilic 8 amino acid sequence that is repeated in tandem 9 times. Immunolocalization indicates that trp is expressed in the rhabdomeres of the photoreceptor cells. Rhabdomeres are specialized membranes of the photoreceptor cells composed of numerous microvilli containing rhodopsin and other components of the phototransduction cascade. The trp protein appears to be missing in each of the mutant alleles analyzed. Thus, the phenotype arises from absence of the protein rather than expression of a defective gene product.

Results

Isolation and Sequence Analysis of trp cDNAs

Two cDNA libraries prepared from mRNA expressed in the head of adult Drosophila were screened with the 6.5 kb trp genomic fragment previously shown to rescue the
trp phenotype by germ line transormation (Montell et al., 1985). Among the positives, was a cDNA, λtrp-9 (3.9 kb), containing the entire protein coding region of trp.

Figure 1 displays the complete sequence of the trp coding region, obtained by DNA sequence analysis of several cDNAs including λtrp-9. Based on the DNA sequence data and primer extension analysis (see below), it appears that the trp mRNA is 4.1 kb. Assuming translation is initiated from the first AUG, trp encodes a protein of 1275 amino acids (or about 143 kd). Comparison of the deduced amino acid sequence with the protein sequence data bank indicated that trp does not fall into any class previously known to be required in signal transduction.

Based on hydrophobicity analysis, according to the algorithm of Kyte and Doolittle (1982), the trp protein can be divided into three domains: a 333 amino acid N-terminal domain with an overall neutral charge, a 228 amino acid central domain with as many as eight putative transmembrane segments, and a C-terminal domain of 614 amino acids with an overall hydrophilic character (Figure 2). The trp protein does not begin with an N-terminal hydrophobic signal sequence. Among the eight putative transmembrane regions in the central domain, segments 2, 3, 5, and 7 have hydrophobicity indices between 1.6 and 2.1 and are the most likely to be membrane-spanning regions. Segments 4, 5, and 7 are somewhat less likely, as their indices are about 1.3. Segment 1 has an index of only about 1.0 due to the presence of 3 charged and 4 polar residues. Using a two-dimensional wheel diagram (Schiffer and Edmundson, 1967), all 7 of these residues would be predicted to fall on the same face of the helix (data not shown). This type of clustering has been proposed to give rise to membrane-spanning amphipathic helices in a number of transport proteins that span the plasma membrane multiple times (reviewed in Catterall, 1988). A total of 15 charged residues are dispersed among the first seven putative membrane-spanning regions. However, the other putative transmembrane domains do not show as strong a clustering of charged and polar residues on one side. Although there are a few other regions with hydrophobicity indices greater than 1.0 (Figure 2), they are all less than 10 amino acids and are too short to be membrane-spanning regions.

The region between amino acids 980 and 1240 in the C-terminal domain is the most hydrophilic. Located near the C terminus is an 8 amino acid sequence, D-K-D-K-K-PG/A-D, repeated in tandem 9 times (underlined in Figure 1 and illustrated in the hydrophobicity plot in Figure 2). Although 6 out of the 8 residues in each repeat are either acidic or basic, the overall charge is neutral. The tripeptide K-P-X (X is most commonly A or G) is repeated a total of 27 times in a 253 amino acid segment encompassing the 8 x 9 repeats.

Structure of the trp Gene
The intron-exon structure of the trp gene was determined by sequencing the 6.5 kb genomic region, which rescued the trp phenotype (Montell et al., 1985), and comparing this sequence with that of the cDNAs (Figure 3). The initiation of transcription was determined by

![Figure 3. Structure of the trp mRNA](image)

The bottom line represents the genomic DNA demarcated in kilobase pairs. The locations of the restriction sites EcoR I (E), EcoR V (EV), Not I (N), Smal (S), Sacl I (S2), and Xho I (X) are shown. The bold horizontal lines joined by the caret symbols represent the exon and introns of the 4.1 kb mRNA. The direction of transcription is indicated by the arrowhead. The 1275 amino acid trp protein is represented by the boxes. The black boxes represent a hydrophobic region, the stippled box a very hydrophilic region, and the diagonally hatched boxes regions that are slightly hydrophilic or have an overall neutral charge.
primer extension analysis (Figure 4). The sequence at the 5' end resembles the consensus sequence, ATCAC\textasciitilde T/T/T, which is found at the transcription initiation sites of many Drosophila genes (Hultmark et al., 1986). The trp mRNA initiates primarily at the third nucleotide in this consensus sequence with a minor start site at the first nucleotide. Near the 3' end of the gene is a typical AATAAA sequence required for 3' end formation (reviewed in Platt, 1986).

The 6.5 kb trp genomic fragment contains only 437 and 317 bp flanking the 5' and 3' ends of the transcribed region. Since this fragment rescued the mutant phenotype (Montell et al., 1985), it appears that less than 450 bp are required upstream or downstream of the transcribed region for proper expression of trp. Beginning 47 bp upstream of the start site of transcription is a perfect match to an 11 nucleotide consensus sequence, CTAAATTCRRTT (underlined in Figure 1), flanking the 5' end of other photoreceptor cell-specific genes (Wismer et al., 1988). The sequence AATAAA (underlined Figure 1), beginning 32 nucleotides from the site of transcription initiation, may serve the same function in trp as a TATA box.

**Immunological Identification of the trp Protein**

To localize the trp protein spatially on cytological sections of an adult fly head and to identify the trp protein on protein immunoblots, mouse polyclonal antisera were prepared to a β-galactosidase–trp fusion protein. The fusion protein, ZETrp, included an approximately 150 amino acid segment immediately N-terminal to the 8 × 9 tandem repeat. To identify the trp protein on immunoblots, extracts were prepared from the heads and bodies of wild-type flies, fractionated on a SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with the trp antisera (aZETrp). A single protein of the size predicted from the deduced amino acid sequence was detected in the extracts of wild-type heads, but not of bodies (Figure 5).

**RNA and Protein Expression from trp Mutant Alleles**

The trp RNA expressed from the three mutant alleles, CM, P301, and P343, is shown in Figure 6. The concentration of the trp RNA was reduced in each of the three alleles analyzed. The greatest decrease was observed in
of the 143 kD band present in the heads of wild-type flies was detected in any of the trp mutants. These results strongly suggest that CM, P301, and P343 are all null alleles.

The trp Protein Is Spatially Localized to the Rhabdomeres

The compound eye of the fruit fly consists of approximately 800 repeat units referred to as ommatidia. Each ommatidium consists of photoreceptor cells, pigment cells, and corneal cells. The photoreceptor cells have a specialized portion of the plasma membrane, the rhabdomeres, consisting of tightly packed microvilli. The rhabdomeres contain high concentrations of rhodopsin and are the site of photoreception. To determine which cells within the compound eye express trp, we performed immunofluorescent localization. The mutant phenotype suggests that trp would be expressed in the photoreceptor cells.

Each ommatidial unit contains 8 photoreceptor cells. Two of the photoreceptor cells, R7 and R8, contain rhabdomeres that occupy the central distal and central proximal regions of the retina, respectively. The other 6 photoreceptor cells extend the full length of the retina and contain rhabdomeres closer to the periphery of the ommatidia. The rhabdomeres of the photoreceptor cells are spatially separated from each other. The photoreceptor cells are surrounded by pigment cells and covered by the cornea. If a protein is distributed evenly throughout the photoreceptor cells, then the immunofluorescence would be expected to appear as one continuous bundle of staining in each ommatidium, since the photoreceptor cell bodies are contiguous. However, if a protein is expressed specifically or predominantly in the rhabdomeres, then the immunofluorescent pattern would not appear as one continuous bundle of staining in each ommatidium, since each rhabdomere is spatially separated from the other rhabdomeres. Instead, the immunofluorescent pattern would appear as ribbons of staining extending the full length of the retina.

Figure 7 shows a 7 μm section of a wild-type adult fly

Figure 6. Analysis of trp RNA from Mutant Alleles
Poly(A)⁺ RNAs were prepared from wild-type adults and three mutant alleles, CM, P301, and P343, fractionated on a 3% formaldehyde, 1.5% agarose gel (2 μg per lane), transferred to nitrocellulose, and probed with pc339-8 nick-translated with 32P. The 4.1 kb size of the trp RNA, indicated in kilobases, is based on the sequences of the cDNAs and primer extension experiments. Taking into account the typical 200 base poly(A) sequence at the 3' end of most mRNAs, the 4.1 kb size matches closely the 4.2 kb estimate relative to DNA size markers (Montell et al., 1985). The RNA blot was reprobed with a Drosophila actin gene, pDMA2 (Fryberg et al., 1983), to demonstrate that equal concentrations of RNA were loaded onto each lane.

P343. The reductions in trp RNA levels were not due to poor RNA transfer, as the concentration of actin RNA was similar in the wild type and in each mutant (Figure 6).

Protein extracts were prepared from the heads and bodies of the same three trp alleles and analyzed by the protein immunoblot procedure (Figure 5). No evidence
nel has an even number of multiple transmembrane domains, one of which displays amphipathic character, and no hydrophobic N-terminal signal sequence (Tanabe et al., 1987). The GABA<sub>A</sub> receptor also has an even number of transmembrane domains and no N-terminal signal sequence (Schofield et al., 1987). These other integral membrane proteins fall into various classes. For example, voltage-activated channels, such as the K<sup>+</sup>, Na<sup>+</sup>, and Ca<sup>2+</sup> channels, all have striking primary amino acid homology (reviewed in Catterall, 1988). The GABA<sub>A</sub> glycine, and nicotinic acetylcholine ligand-gated receptors form another family of highly related proteins (reviewed in Barnard et al., 1987). The individual members of each family show no specific amino acid homology with the members of another family. However, they all share the common features of an even number of transmembrane segments and the absence of a signal sequence. It is possible that trp is the first member of another general class of integral membrane proteins sharing these general features with receptor/transport proteins and having a role in signal transduction.

According to the model shown in Figure 8, the trp protein has an even number of transmembrane segments. If this model is correct, then the N and C termini must lie on the same side of either the rhodopsin or vesicular membranes. The ends of the trp protein are depicted on the cytoplasmic side, as there is no N-terminal signal sequence. The localization of the trp protein to the membrane-rich rhodoseres provides additional evidence that trp is an integral membrane protein.

The Structure and Localization of the trp Protein Supports Certain Possible Functions and Disfavors Others

The trp mutation is characterized by a rapid decay of the receptor potential during illumination with bright light and by a requirement for about a 60 s recovery period in the dark before a subsequent full response to light can be elicited. Electrophysiological and pharmacological studies suggest that trp has a role subsequent to the production of IP<sub>3</sub> (Suss et al., 1989). Indirect evidence suggests that the intracellular Ca<sup>2+</sup> levels may be reduced in trp photoreceptor cells (Zuidervaart et al., 1979, Soc. Neurosci., abstract; Kirschfeld and Vogt, 1980; Lo and Pak, 1981; Minke, 1982). In addition, the nss and trp mutations appear to lower the activity of the PLC, suggesting that trp may be involved in feedback control of the phototransduction cascade (Suss et al., 1989). This latter effect may be a consequence of the decreased intracellular Ca<sup>2+</sup> levels in trp, since the activity of PLC is dependent on Ca<sup>2+</sup> concentration (Baer and Saibil, 1988). Based on these observations, there are many potential roles for trp. The results from the current paper lead us to disfavor most of these possibilities and focus on potential roles that are most consistent with the molecular analysis: a role in inositol phospholipid metabolism or possibly as a membrane protein that interacts with the light-sensitive ion channels.

Subsequent to the production of IP<sub>3</sub>, other inositol phosphates such as IP<sub>4</sub>, IP<sub>5</sub>, and IP<sub>6</sub> are produced (re-
viewed in Downes, 1988). Little is known about the functions of these components or the proteins that synthesize them. Several studies have led to the suggestion that IP₃ may act as a second messenger. One proposal is that IP₃ facilitates Ca²⁺ entry into the cytoplasm by opening channels in the plasma membrane (Irvine and Moor, 1986). Another study has raised the possibility that IP₃ and IP₄ may act synergistically to regulate ion channels in the plasma membrane and evoke a full electrophysiological response (Morris et al., 1987). Consistent with the spatial localization and putative structure is the possibility that trp is the plasma membrane IP₃ receptor. Absence of the plasma membrane IP₃ receptor in trp could result in decreased intracellular Ca²⁺ levels, leading to lower Ca²⁺ levels in the internal storage vesicles. Rapid depletion of the small internal Ca²⁺ stores could account for the loss of receptor potential during bright illumination and the observation that the trp mutant has a defect subsequent to the production of IP₃. Lower total cytoplasmic Ca²⁺ levels could be responsible for the decreased PLC activity, since this enzymatic activity is affected by Ca²⁺ concentration.

In addition to IP₃, it appears that IP₄ and IP₈ also have physiological functions in the nervous system (Vallejo et al., 1987). It is possible that inositol phosphates, such as IP₂, IP₃, or IP₄, play a role in Drosophila phototransduction and that trp is important for production of one of these components. The trp protein might be an inositol phosphate kinase involved in the production of one of these higher phosphorylated forms of inositol. Although the trp protein has eight putative transmembrane domains, the majority of the protein, 333 and 614 amino acids at the N and C termini, is predicted to extend into the cytoplasm. An inositol kinase activity may be contained in these large terminal domains. The recent study has demonstrated that IP₄ induces Ca²⁺ sequestration in rat liver cells (Hill et al., 1988). It is possible that IP₄ plays a similar role in Drosophila photoreceptor cells. Thus, failure to produce IP₄ could result in the trp phenotype either by limiting entry of Ca²⁺ into the photoreceptor cells or by impairing Ca²⁺ sequestration into the internal stores.

Alternatively, the trp protein could interact with the light-sensitive channels in the plasma membrane. There is evidence that the properties of channels can be modified by interaction with other proteins (see for example Ganetsky and Wu, 1986). The trp protein might have a role in reactivation of the light-sensitive channels in photoreceptor cells. Wild-type flies continually display a response to light and do not require a recovery period in the dark even after a very long and intense light stimulus. This is in contrast to the trp mutant, which quickly loses responsiveness to bright light and requires a 60 s dark recovery period before a subsequent intense light stimulus can elicit a full depolarization. It is possible that the loss of responsiveness during intense illumination and the 60 s dark recovery period are due to a requirement for the trp protein to quickly reset the light-sensitive channels before they can interact again with ligand and/or reopen. The trp protein may not be required under dim light conditions, as only a fraction of the light-sensitive channels are activated at any time and these could reset slowly as other channels are activated. However, the limitation of this model is that it does not account for the diminished PLC activity in trp.

The molecular analyses presented in the current paper argue against certain other possible functions for trp. For example, the trp protein might be an IP₃ receptor. Binding of IP₃ to IP₃ receptors has been shown to lead to the release of intracellular Ca²⁺ (Meyer et al., 1988). Alternatively, since trp is required subsequent to the production of IP₃ and the intracellular Ca²⁺ levels appear to be reduced, it is possible that trp has a role in storing Ca²⁺ (Suss et al., 1989). Therefore, trp might encode a Ca²⁺-ATPase that pumps Ca²⁺ into the internal stores or a Ca²⁺ channel that releases Ca²⁺ from the storage vesicles. However, the trp protein is localized predominantly to the rhabdome, the specialized plasma membrane of the photoreceptor cells, and does not appear to be specifically localized to the membranes of any intracellular vesicle. Additionally, the trp protein is not homologous to the mammalian sarcoplasmic reticulum Ca²⁺-ATPase (MacLennan et al., 1985; Brandt et al., 1986) and is much smaller than the 260 kd IP₃ receptor purified from rat brains (Supattapone et al., 1988) and the 400 kd Ca²⁺ release channel from the vertebrate sarcoplasmic reticulum (Lai et al., 1988).

Since trp is localized to the rhabdome, it is possible that it is a plasma membrane Ca²⁺ pumping ATPase. However, trp shows no homology with the recently reported C-terminal sequence of a plasma membrane Ca²⁺-ATPase (Brandt et al., 1988). In particular, the trp protein does not contain any sequence that matches the 6 key residues in the 12 amino acid Ca²⁺ binding loops originally identified in calmodulin (Waterson et al., 1980) and subsequently in the plasma membrane and sarcoplasmic reticulum Ca²⁺-ATPases.

An alternative proposal, consistent with the protein structure and localization, is that trp is the structural gene for the light-sensitive channels. However, electrophysiological analyses suggest that trp does not encode the light-sensitive channel (Minke, 1982). This conclusion is strongly supported by the protein immunoblots analysis of three trp alleles presented in the current report. Since the behavioral and electrophysiological response of trp flies is normal under conditions of dim light, the light-sensitive channels must be present. Therefore, if trp encodes the light-sensitive channel, then the protein must be defective rather than absent. The demonstration that the trp protein is completely missing in each mutant allele examined, indicates that trp is not the structural gene for the light-sensitive channel.

In conclusion, on the basis of the spatial localization, the putative protein structure, and the analysis of protein expression from several mutant alleles, it appears that many potential roles for trp can now be considered highly unlikely. Thus, it appears that trp is a protein with a role subsequent to the production of IP₃ that has not
been previously described. The results presented in the current paper are consistent with a role in the inositol phospholipid signaling system.

Experimental Procedures

RNA Analyses
Polyadenylated RNA was prepared from wild-type adult flies (Oregon R strain of D. melanogaster) and from the mutant alleles CM, P301, and F343 as described (Montoil et al., 1985). CM is the original allele isolated (Coxens and Manning, 1969), and the P301 and F343 alleles were isolated by W. L. Pak. Polyadenylated RNA (2 µg of each) wasfractionated on 3% formaldehyde, 1.5% agarose gels as previously described (Montoil et al., 1985). The RNAs were transferred to nitrocellulose by blotting and then probed with pC559-8 (a trp cDNA; see below) nick-translated with 32P.

Primer extensions were carried by hybridizing a synthetic oligonucleotide (nucleotides 234–250; Figure 4i) 5’ end-labeled with 32P with 3 µg of head or body poly(A)* RNA from the Oregon R strain. Reverse transcription was carried out as described (Maniatis et al., 1982) for cDNA synthesis. The synthetic oligonucleotide was also used to obtain a DNA sequencing ladder (for size markers) after hybridization with an M13–trp genomic clone, mp33D1. This M13 clone was created by inserting the 0.8 kb EcoRI–XhoI fragment encompassing the 5’ end of trp (Figure 3) between the EcoRI and SalI sites of M13mp18.

Isolation of cDNAs
A cDNA library prepared from D. melanogaster Oregon R strain adult head poly(A)* RNA (gift from B. Yedrobovick and S. Artavan-Sakonas) was screened with 32P-labeled pS59f1.7 (Montoil et al., 1985). pS59f1.7 consists of a 1.6 kb genomic EcoRI fragment from the 3’ end of trp (4.9–6.5 kb; see Figure 3). The filters were hybridized and washed as described (Montoil et al., 1987). Among the cDNAs isolated, c559-8 was the longest (nucleotides 585–4066; Figure 1). DNA sequence analyses of several other trp cDNAs, as well as the genomic DNA, showed that c559-8 is missing 2 nucleotides (1631–1632; Figure 1).

A second Drosophila cDNA library, prepared from adult head poly(A)* RNA (Cowan and Rubin, unpublished data), was subsequently screened with pS59C3A to isolate longer cDNAs. pS59C3A contains the 6.5 kb trp genomic region that rescued the trp phenotype (Montoil et al., 1985). One of the cDNAs isolated, mp399, includes the entire protein coding region. The cDNA that extends closest to the 3’ end, mp399 (3.8 kb), begins at nucleotide 77 but terminates approximately 100 nucleotides before the 3’ end. Each of the cDNAs mapped by in situ hybridization to polytene salivary gland chromosomes to the cytogenetic position of trp, 99C (Levy et al., 1982; Wong et al., 1985; Montoil et al., 1985).

DNA Sequencing
DNA sequencing was carried out according to the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (United States Biochemicals, P57,5ATP) and 60 cm buffer gradient gels (Biggin et al., 1983).

The plasmid pC559-8 was constructed by partial digestion of λS59-8 with EcoRI and subcloning the 3.5 kb fragment into the EcoRI site of pEMBL9 (Dente et al., 1983). The λcrrp-9 cDNA consists of a 2.7 and a 1.3 kb EcoRI fragment (the 1.3 kb fragment includes a 3’ poly(A) tail of about 100 bases). The λcrrp-7 cDNA consists of a 2.8 and a 1.1 kb EcoRI fragment. Both λcrrp-9 EcoRI fragments and the 2.8 kb λcrrp-7 fragment were subcloned into the EcoRI site of Bluescript KS M13(−) (Stratagene) to create the plasmids pcppr-9.2-7, pcppr-9.1-3, and pcppr-9.7. The trp fragments in the plasmids pcppr-9.2-7 and pcppr-9.1-3 have the 5’ and 3’ ends (with respect to the orientation of transcription) closest to the universal primer. The 2.8 kb fragment is inserted in pcppr-9.7 with the 3’ end closest to the M13 – 20 primer (with respect to the orientation of transcription). The plasmid pS59C3A (Montoil et al., 1985) consists of the 6 kb genomic fragment, which rescued the trp mutation. subcloned into the transformation vector Carnegie 3 (Rubin and Spradling, 1983).

Ten micrograms of pc559-8 and 10 µg of pS59C3A were randomly sheared by sonication and repaired, and fragments in the 300–600 nucleotide size range were inserted into the Smal site of M13mp10 as described (Montoil et al., 1987). Recombinant M13 clones containing the cDNA inserts were identified by plaque hybridizations with the corresponding purified fragments nick-translated with 32P. The inserts were sequenced using the universal primer (New England Biolabs #1211). Single-stranded pcppr-9.2-7 and pcppr-9.1-3 DNA's were prepared and sequenced using a series of chemically synthesized oligo nucleotides spaced 300 nucleotides apart. The 5’ end of pcppr-7 was sequenced using single-stranded pcppr-7R and the same oligonucleotide used for the primer extension (nucleotides 234–250).

Preparation of trp Antiserum
The trp antiserum was raised to a protein consisting of β-galac-
toside joined at the C terminus to a fragment of the trp protein. The fusion protein was generated using the β-galactosidase expression vector, pUR292 (Ruther and Müller-Hill, 1983). To generate the plasmid pE373, pUR292 was digested with an excess of BamHI, partially digested with EcoRI, and ligated to a 0.35 kb BamHI–EcoRI fragment from M13c559-8-100 (beginning at nucleotide 3180; Figure 4i). M13c559-8-100 was generated by random shearing of c559-8 and used in the DNA sequence analysis (see above). The fusion protein was expressed in E. coli K12 strain BKM 71-18 and purified by electrophoresis from an SDS–polyacrylamide gel as described (Montoil and Rubin, 1988). Samples (100 µl per mouse; 0.5–0.5 µg of protein) were emulsified by sonication in 200 µl of Freund's complete adjuvant and introduced subcutaneously into two BALB/c female mice. The mice were subsequently injected after 3 and 6 weeks with similar quantities of the fusion protein emulsified in Freund's incomplete adjuvant. Seven days following the last boost, the animals were bled and sera (α2-E2trp) were collected.

Protein Blot Analysis
Sixteen milligrams of adult heads and bodies, separated in mass by agitation of frozen flies (Olliver and Phillips, 1970), was emulsified in 400 µl of 2 x SDS sample buffer using a microfuge tube pellet pestle (Kontes Scientific) and a motor-driven homogenizer (Belco Glass Inc.). The extracts were boiled for 2 min, and 10 µl of each protein extract was fractionated on an SDS–8% polyacrylamide gel. The proteins were transferred to nitrocellulose by electrophoresis in transfer buffer (20 mM Tris base, 150 mM glycine, 20% v/v methanol) without SDS. The nitrocellulose was rinsed briefly in PBS, incubated at room temperature for 4 hr in PBS–BSA (5% BSA dissolved in PBS; pH 7.4) rinsed in PBS, incubated overnight at 4°C with α2-E2trp from mouse #8 (diluted 1500 in PBS–BSA), washed for 10 min in PBS, washed twice for 10 min in PBS with 0.05% NP-40, and rinsed once in PBS. The nitrocellulose was then incubated for 4 hr at room temperature with 0.2 µCi 125I–labeled sheep anti-mouse immunoglobulin (Amersham) in PBS–BSA, washed as above, and exposed to autoradiography.

Light Microscopy Immunolocalization
Frozen sections (7 µm) of adult fly heads (w1118) were mounted on slides previously dipped in 0.1 mg/ml poly-l-lysine, dried, fixed for 30 min with 2% formalin in 75 mM sodium phosphate (pH 7.0), and rinsed for 10 min in TBS (10 mM Tris–HCl (pH 7.4), 130 mM NaCl, 5 mM KCl, 1 mM EDTA). The fixed sections were covered for 30 min at room temperature with 50 µl of 50% fetal bovine serum (blocking serum) over a humidified chamber, rinsed for 10 min in TBS covered overnight at 4°C with 50 µl of α2-E2trp (1:250 dilution in TBS), rinsed for 10 min in TBS, and covered for 30 min at room temperature with 50 µl of rhodamine-conjugated, affinity-purified goat anti-mouse IgG (Cappel Worthington) diluted 1:50 in TBS. The sections were then rinsed for 10 min in TBS, covered with 50 µl of mounting medium (90% glycerol, TBS, 25 mM propyl gallate) and a coverslip and viewed by epifluorescence.
Acknowledgments

We thank Drs. B. Minke, D. Ruben, J. Porter, and R. Moorey for their helpful comments on this manuscript, Dr. W. L. Pak for providing stocks of several of the tip mutant alleles, and Todd Lavery for performing in situ hybridizations to salivary gland polytene chromosomes. This work was supported in part by the Howard Hughes Medical Institute and by an NIH postdoctoral fellowship to C. M.

Received January 11, 1989; revised February 16, 1989.

References


