Nonsense Suppression of the Major Rhodopsin Gene of Drosophila

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ABSTRACT

We placed UAA, UAG and UGA nonsense mutations at two leucine codons, Leu_{205} and Leu_{309}, in Drosophila's major rhodopsin gene, *ninaE*, by site-directed mutagenesis, and then created the corresponding mutants by *P* element-mediated transformation of a *ninaE* deficiency strain. In the absence of a genetic suppressor, flies harboring any of the nonsense mutations at the 309 site, but not the 205 site, show increased rhodopsin activity. Additionally, all flies with nonsense mutations at either site have better rhabdomere structure than does the *ninaE* deficiency strain. Construction and analysis of a 3′-deletion mutant of *ninaE* indicates that translational readthrough accounts for the extra photoreceptor activity of the $ninaE^{309}$ alleles and that truncated opsins are responsible for the improved rhabdomere structure. The presence of leucine-inserting tRNA nonsense suppressors DtL^a Su^+ and DtL^b Su^+ in the mutant strains produced a small increase (less than 0.04%) in functional rhodopsin. The opal (UGA) suppressor derived from the DtL^a tRNA gene is more efficient than the amber (UAG) or opal suppressor derived from the DtL^b gene, and both DtL^a and DtL^b derived suppressors are more efficient at site 205 than 309.

THE deleterious effects of nonsense mutations are ameliorated by second, nonallelic mutations in genes encoding components of the translation machinery. Genetic screens for informational suppressors often result in isolating strains harboring altered tRNAs (WILLS *et al.* 1983). Usually, the anticodons of the tRNAs are mutated, enabling them to insert amino acids at amber (UAG), opal (UGA) or ochre (UAA) nonsense mutations. Genetic nonsense suppressors produce a range of suppression efficiencies. In *Caenorhabditis elegans*, certain suppressors have been shown to be more efficient in one tissue than another, perhaps because these tRNA genes are variably expressed between tissues (HODGKIN 1985; KONDO, HODGKIN and WATERSTON 1988; KONDO *et al.* 1990). Nonsense mutations at different sites in the same gene are often not equally suppressed by the same nonsense suppressor. This has been explained by mRNA context effects, where the nucleotide context flanking the different sites plays a role in the efficiency of their suppression (BOSSI 1983; BOSSI and ROTH 1980; MILLER and ALBERTINI 1983).

Genetic screens have been successfully used to find tRNA nonsense suppressors in *Escherichia coli*, yeast and *C. elegans*, but not in Drosophila (KUBLI 1982). Drosophila strains harboring nonsense suppressor tRNAs have been made by site-directed mutagenesis and *P* element-mediated transformation of cloned tRNA genes (DOERIG *et al.* 1988; GARZA, MEDHORA and HARTL 1990; LASKI *et al.* 1989). Suppression efficiencies *in vivo* of these strains were less than 1%, and only for one endogenous gene tested, an amber allele of rosy (DOERIG *et al.* 1988), was a phenotypic difference between suppressed and unsuppressed flies detected. The other gene shown to be suppressed *in vivo* was a transformed amber allele of the bacterial chloramphenicol acetyl transferase gene, and this suppression efficiency was only 0.4% (GARZA, MEDHORA and HARTL 1990; LASKI *et al.* 1989). Therefore, detecting suppression in Drosophila has been possible only when assays are sufficiently sensitive to detect very low levels of a gene product. To compare the efficiencies of different tRNA suppressors or the efficiency of a single suppressor at different nonsense mutation sites within a gene, the assay system should be able to differentiate between gene product levels less than 0.4% of wild type.

We constructed nonsense mutations in the *ninaE* gene, which encodes the rhodopsin expressed in photoreceptor cells R1–6 of the Drosophila eye (O'TOUSA *et al.* 1985; ZUKER, COWMAN and RUBIN 1985), and used the visual response of the fly as an assay for testing the suppression of these mutations. Electrophysiological assays are sensitive indicators of rhodopsin activity levels, since the amplification of the response is such that 1 photoactivated rhodopsin molecule produces a discrete membrane depolarization or "quantum bump" as observed by intracellular recordings (JOHNSON and PAK 1986). A technically simpler identification of rhodopsin activity, using extracellular recordings (electroretinogram, ERG), is the response of second order neurons to photoreceptor cell activity. R1–6 photoreceptor cells synapse in the lamina, and neurons of the lamina respond to R1–6 photoreceptor

activity with on- and off-transients which are readily observed in the ERG. R7 and R8 cells express other opsins and synapse in the medulla, the second optic ganglion. The activity of the medulla does not obviously affect the ERG. The light intensity necessary to elicit an off-transient in an ERG is a relative measure of functional rhodopsin present in R1–6 cells, and flies with as low as 0.08% functional rhodopsin (JOHNSON and PAK 1986) are able to produce off-transients.

Null mutations in the *ninaE* gene cause defects in photoreceptor cell structure and physiology. $ninaE^{ol17}$ flies contain a large deletion in the 5′-region of the gene and make no detectable *ninaE* transcript (O'TOUSA *et al.* 1985), and therefore, $ninaE^{ol17}$ is a null allele. The lack of rhodopsin expression in R1–6 cells of $ninaE^{ol17}$ flies results in the loss of R1–6 cell rhabdomeres, the organelles into which rhodopsin is packed, while rhabdomeres of R7 and R8 cells, which express different opsins, remain intact (O'TOUSA, LEONARD and PAK 1989; STARK and CARLSON 1983). The lack of R1–6 cell rhodopsin also results in an absence of transient elements in the ERG and a reduced ERG amplitude. A *ninaE* nonsense mutant, $ninaE^{ora}$, shows similar defects in photoreceptor cell structure and physiology (O'TOUSA, LEONARD and PAK 1989; STARK and CARLSON 1983). Photoreceptor cell ultrastructure and ERG improvements from these examples of the null state indicate increased R1–6 cell rhodopsin content.

Here we report using electrophysiology and photoreceptor cell morphology to assess the effects of suppression of *ninaE* nonsense mutations. We generated nonsense mutations at different sites in the *ninaE* gene and compared the relative suppression efficiencies in strains lacking a genetic suppressor to those carrying nonsense suppressors derived from two leucine-inserting tRNA genes (GARZA, MEDHORA and HARTL 1990).

MATERIALS AND METHODS

Site-directed mutagenesis: *Kpn*I linkers were placed by C. ZUKER (University of California, San Diego) on the 5.5-kb *Hin*dIII fragment containing the wild-type *ninaE* gene. We cloned this *Kpn*I fragment into pUC118, and the resulting plasmid was used to transform *E. coli* strain BW313 (KUNKEL, ROBERTS and ZAKOUR 1987). Single-stranded templates were produced according to VIEIRA and MESSING (1987), except that uridine was added at 0.25 μg/ml to the growth medium. Site-directed mutagenesis was performed as described (KUNKEL, ROBERTS and ZAKOUR 1987). Oligonucleotides of the sequences CCTCGTGCGGTATTGACTAC**TRR**GAACGCGACTGGAACCC and CGAGGGCCTCACACCA**TRR**AACACCATTTGGGGAGCTTGC span the nucleotides corresponding to leucine codons 205 and 309, respectively. The bold type indicates the codons being mutated, and each R represents a mix of adenine and guanine nucleotides. Reactions using these mixed primers produced each of four mutant sequences: UAA (ochre nonsense), UAG (amber nonsense), UGA (opal nonsense) and UGG (tryptophan missense). An oligonucleotide of the sequence GGGCCTCACACCATGA*TAAATTCTTTGGCGC was used to produce a deletion in the *ninaE* gene. The asterisk labels the site of the 260-nucleotide deletion and the underlined triplets are the 309 site opal termination codon and the endogenous termination codon, respectively. The mutations were confirmed by single stranded (VIEIRA and MESSING 1987) dideoxy sequencing (SANGER 1981) using the sequenase kit (U.S. Biochemical).

Subcloning and Drosophila transformations: Each 5.5-kb *Kpn*I fragment containing a mutated *ninaE* gene was subcloned into the P element transformation vector Carnegie3 rosy2 (RUBIN and SPRADLING 1983). This vector contains a wild-type rosy gene to assay *P* element integration into the Drosophila genome. Flies of the genotype ry^{506} $ninaE^{ol17}$ e^{s} were the DNA recipients in standard transformation experiments. Transformant lines were made homozygous for the P element insert and the *X* chromosome mutation *white*.

RNA analysis: Total RNA from Drosophila heads was isolated according to CATHALA *et al.* (1983). Approximately 6 μg of RNA was electrophoresed on a 1.5% agarose, 6% formaldehyde gel, blotted onto nylon filter paper (Amersham), and probed with either an eye-specific cDNA clone (*dgq*; LEE *et al.* 1990) or with rp49 (O'CONNELL and ROSBASH 1984), and then stripped and reprobed with *ninaE* sequences. The hybridization conditions were as described in CHURCH and GILBERT (1984). The autoradiograms were analyzed by laser densitometry to quantify mRNA levels in the wild-type and mutant lanes.

Histology: Heads of 2-day-old white eyed flies were removed from the bodies, bisected and fixed for electron microscopy. We performed a modification of the BAUMANN and WALZ (1989) fixation protocol. Bisected heads were prefixed in 0.75 M Na cacodylate pH 7.4, 2% paraformaldehyde, and 2% glutaraldehyde 4 hr at room temperature. This was replaced by the same fixative containing 1% tannic acid and incubated overnight at 4°. Three 10-min washes in 0.1 M Na cacodylate, pH 7.4, were followed by postfixation in 0.1 M Na-cacodylate plus 2% osmium tetroxide 2 hr at room temperature. The heads were washed in distilled water 3 times for 10 min each and dehydrated in an ethanol series: 50, 70, 80, 90 and 100% (3 times) for 5 min each. The heads were immersed in a 1:1 solution of xylene to ethanol, 100% xylene, a 3:1 solution of xylene to Poly/bed 812 (Polysciences, Inc.; mixed according to the manufacturer's instructions), and a 1:1 solution of xylene to Poly/bed 812 for 30 min each. The samples were then immersed in 100% Poly/bed 812 overnight at room temperature. Individual eyes were embedded in 100% Poly/bed 812, left at 35° overnight, 45° the next day, and 60° overnight.

Deep pseudopupil assay: The deep pseudopupil assay is described in FRANCESCHINI and KIRSCHFELD (1971). In this assay, red eyed flies are placed between a bright light source and the microscope objective, such that rhabdomeres act as waveguides and appear bright relative to the rest of the eye. The eye's curvature and precisely arranged ommatidia produce a single large virtual image of an ommatidial unit beneath the eye's surface.

ERG off-transient analysis: Two-day-old white eyed flies were subjected to ERG analysis as described previously (*e.g.*, LARRIVEE *et al.* 1981). Flies were given a minimum of 15 min to recover from etherization before beginning the ERGs. The criterion for determining whether a fly exhibits off-transients was the following. Each fly was given 20 1-sec flashes at a single light intensity over a period of 1 min. If at least ten of these flashes elicited an off-transient, then the

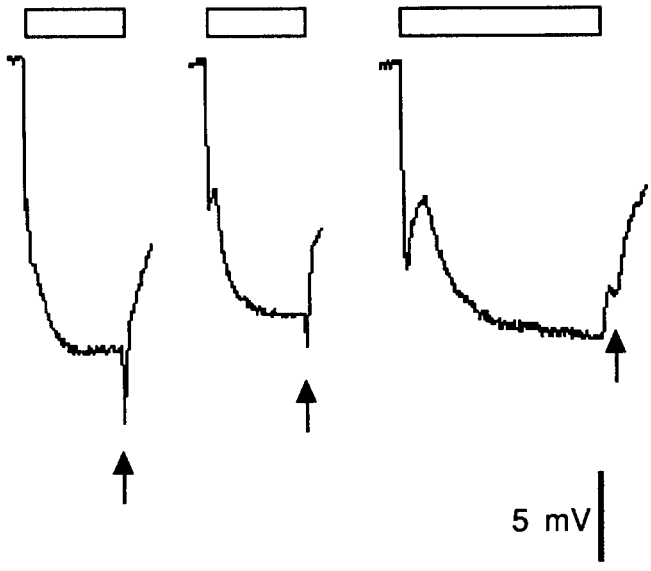


FIGURE 1.—ERG off-transients. The appearance of off-transients varied from large (left) to small and delayed (right). The box above each trace represents a one-second light stimulus, and an arrow indicates each off-transient. In this assay, the dimmest light intensity for which off-transients could be elicited from a given fly often elicited those of the small, delayed type. Brighter lights typically produced larger transients coincident with light-off.

fly was registered as positive for producing off-transients. Progressively dimmer lights were tested until an intensity elicited zero off-transients from the specimen. Log relative intensities of 0, which is the brightest, −0.6, −1.0, −1.6, −2.0, −2.6 and −3.0 were used in this analysis. The brightest white light intensity is approximately 1000 foot-candles (measured by a footcandle/lux meter, Extech Instruments). In our study, an off-transient was defined as a quick, depolarizing response occurring at the end of a light stimulus. Figure 1 depicts the range of depolarizing responses that we considered to represent off-transients. Using this protocol $ninaE^{o117}$ in certain genetic backgrounds produced off-transients with some frequency in response to the brightest light. Since $ninaE^{o117}$ contains a large deletion at the 5′-end of the gene (O'TOUSA *et al.* 1985) and makes no detectable transcript, the transients elicited from $ninaE^{o117}$ flies with the bright light must not be due to *ninaE* gene product function.

ERG amplitudes: A 1-sec white light stimulus (0 intensity) was used to elicit photoreceptor responses from flies. Flies were dark-adapted for 30 sec prior to recording ERGs. ERG amplitudes, as plotted and measured by the Unkelscope software package, were calculated by subtracting the voltage measured just prior to light stimulation from the voltage measured 0.5 sec into the stimulus.

Suppressor stocks: The suppressor stocks used here were derived from transformant lines generously provided by GARZA, MEDHORA and HARTL (1990): (1) $b\ Adh^{n4}$ P[ry^+, DtL^b Su^+ (amber)]/SM1; ry^{506} $ninaE^{o117}$ e^s, (2) P[ry^+, DtL^bSu^+ (opal)] /SM1; ry^{506} $ninaE^{o117}$ e^s, and (3) P[ry^+, DtL^a Su^+ (opal)]/SM1; ry^{506} $ninaE^{o117}$ e^s. The first stock is derived from the D7 line described in GARZA, MEDHORA and HARTL (1990). These authors also constructed the opal suppressor of the second and third stocks, but did not give them specific designations in the published report. The *P* element construct in the second stock was originally located on the *X* chromosome, but was moved to the second chromosome in our laboratory using the Δ2-3 source of transposase (ROBERTSON *et al.* 1988).

Crosses: To minimize genetic background variations, ERG and histological analyses were performed on the white-eyed progeny of crosses between homozygous *ninaE* nonsense mutant flies and $ninaE^{o117}$ flies (the same stock used in the transformation experiments). ERG and histological comparisons of suppressed and unsuppressed flies were performed on white-eyed progeny of crosses between suppressor stock flies (listed above) and homozygous *ninaE* nonsense mutant flies. Half of these progeny carry the suppressor and half carry the balancer chromosome (unsuppressed). Thus, comparisons of suppressed and unsuppressed flies are made between siblings that were reared in the same vial and shared similar genetic backgrounds.

RESULTS

Amber (UAG), opal (UGA) and ochre (UAA) stop codons were inserted into the coding region of the wild-type *ninaE* gene via site-directed mutagenesis. Each replaced a leucine codon, identified as Leu_{205} or Leu_{309}, of the opsin gene. The model in Figure 2 depicts the location of Leu_{205} and Leu_{309} within rhodopsin. The truncated opsins translated from these constructs should not be able to initiate phototransduction since they are missing the retinal binding site (Lys_{319}; O'TOUSA *et al.* 1985). The mutated genes were cloned into a *P* element transformation vector (RUBIN and SPRADLING 1983), and each was used to transform flies carrying a deletion in the endogenous *ninaE* gene ($ninaE^{o117}$; O'TOUSA *et al.* 1985). The levels of *ninaE* mRNA in the transformed lines was assessed by filter hybridization analysis (Figure 3). The steady-state mRNA levels of the nonsense mutants ranged from 30 to 130% of wild type. Another nonsense mutant described in this study, $ninaE^{ora}$ (UAG, see Figure 2 for site of mutation), has 10–20% wild-type *ninaE* mRNA levels.

Rhabdomere morphology: Cross-sections of ommatidia from wild type, $ninaE^{o117}$, and two opal alleles of *ninaE* are shown in Figure 4. $ninaE^{205UGA}$ and $ninaE^{309UGA}$ (Figure 4, C and D) R1–6 rhabdomeres are large relative to the deletion strain, the outer rhabdomeres of which are nearly absent (Figure 4B). However, the nonsense mutant rhabdomeres are smaller than those of wild type (Figure 4A). The central rhabdomere, R7, contains a different rhodopsin (MONTELL *et al.* 1987; ZUKER *et al.* 1987) and is normal in all *ninaE* mutants.

Rhabdomere integrity can be assessed quickly in a large sample of flies by the deep pseudopupil assay (see MATERIALS AND METHODS). The pseudopupil appearance of each nonsense mutant was classified as falling into one of three types (Figure 5): wild type (outer rhabdomeres are bright and distinct), similar to $ninaE^{o117}$ (outer rhabdomeres are not visible), and an intermediate phenotype (outer rhabdomeres are less bright and distinct compared to the central rhabdomere). Flies were approximately 2 days old and

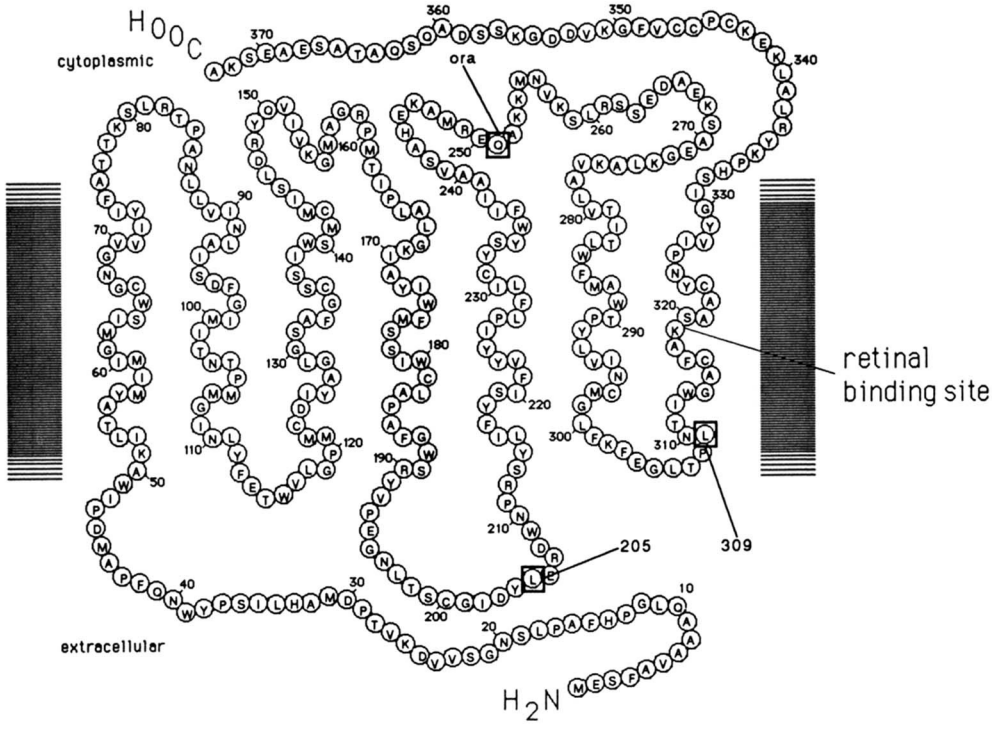


FIGURE 2.—Two-dimensional model of the *ninaE*-encoded opsin. The opsin is shown traversing the membrane seven times. The amino acid positions targeted by the site-directed mutagenesis experiments are indicated by boxes. Leu_{205} and Leu_{309} codons were mutagenized to all three stop codons, the effect of which is to truncate the opsin to 204 and 308 amino acids in length, respectively. Another nonsense mutant, $ninaE^{ora}$, changes a glutamine codon to an amber nonsense codon (WASHBURN and O'TOUSA 1989). The truncated opsins do not contain the retinal binding site, Lys_{319}, and are predicted to be nonfunctional.

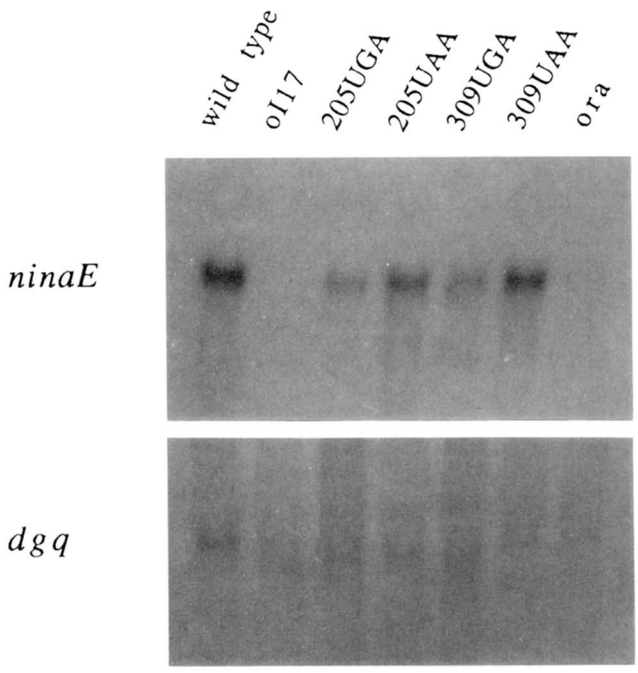
homozygous for the nonsense alleles. By this analysis, $ninaE^{205UGA}$, $ninaE^{205UAG}$, $ninaE^{205UAA}$ and $ninaE^{309UAA}$ are similar to wild type, while $ninaE^{309UGA}$ appears similar to $ninaE^{o117}$, and $ninaE^{309UAG}$ has an intermediate phenotype. Therefore, all the nonsense mutants examined here, including $ninaE^{309UGA}$ (Figure 4D), have better rhabdomere phenotypes than $ninaE^{o117}$ flies.

Electrophysiological phenotype: ERGs (PAK 1979), multicomponent whole eye responses to light, were used to assess the photoreceptor activity of the mutants. At the onset of a light stimulus, the ERG response begins with a short hyperpolarizing on-transient followed by a depolarizing sustained component, and then light-off evokes a depolarizing off-transient. The lack of R1–6 cell activity results in ERG defects in $ninaE^{o117}$ flies. The ERG is small in amplitude, indicative of only R7 and R8 photoreceptor cell function. The ERG also lacks the transient components of the ERG because the transients are produced by secondary neurons of the lamina (COOMBE 1986) in response to the activity of the R1–6 photoreceptors. We found that we could induce off-transients in the ERG of the nonsense alleles described above, showing these mutants must have more R1–6 photoreceptor activity than the $ninaE^{o117}$ allele. However, no light intensity elicited on-transients from the nonsense mutants.

Because the off-transient was a useful phenotype for rapidly assessing R1–6 photoreceptor activity, a protocol was developed to measure the capacity of the nonsense mutant strains to produce off-transients in response to a given light intensity (for details see MATERIALS AND METHODS). Two *ninaE* alleles were used for comparison. $ninaE^{o117}$, a *ninaE* null, is expected to have no R1–6 rhodopsin. $ninaE^{P332}$, a missense mutant (WASHBURN and O'Tousa 1989), has 0.08% of the wild-type levels of functional rhodopsin (JOHNSON and PAK 1986). In order to minimize genetic background effects, nonsense mutant and $ninaE^{P332}$ flies were backcrossed to $ninaE^{o117}$ flies, and their white-eyed progeny were used for the analysis. Therefore, the $ninaE^{P332}$ progeny have 0.04% R1–6 rhodopsin activity. Seven of ten of the $ninaE^{P332}$ progeny responded with transients at the −2 light intensity, while $ninaE^{o117}$ flies derived from the transformation stock produced no off-transients at any intensity (Figure 6A).

The activity of the nonsense alleles falls between that of $ninaE^{o117}$ and $ninaE^{P332}$. In this assay, $ninaE^{205}$ alleles often responded to the brightest light (0 intensity), but not to dimmer lights (Figure 6B). $ninaE^{309}$ alleles have more physiological activity than $ninaE^{205}$; $ninaE^{309UAG}$, $ninaE^{309UGA}$ and $ninaE^{309UAA}$ flies produced transients at the −0.6 intensity, while the latter two also produced transients at the −1 intensity (Figure 6C). The production of off-transients is sensitive to genetic background. When the nonsense mutants were crossed such that their progeny contained the SM1 balancer chromosome (see Figure 9) they responded with off-transients to slightly dimmer lights. Nonetheless, in the genetic backgrounds tested, $ninaE^{309}$ alleles showed more R1–6 activity than $ninaE^{205}$ alleles.

Because the $ninaE^{205}$ and $ninaE^{309}$ nonsense alleles



ninaE transcript levels

dgq	1.0	0.0	0.3	1.0	0.6	1.2	0.2
rp49	1.0	0.0	0.4	1.0	0.7	1.3	0.1

FIGURE 3.—*ninaE* transcript levels from wild-type and mutant flies. Approximately 6 μg of total head RNA from each *ninaE* stock was loaded per lane. The northern blot was probed with *dgq* sequences (LEE *et al.* 1990), stripped, and reprobed with *ninaE* sequences. The autoradiogram bands were quantified using an LKB Ultrascan XL laser densitometer. The *dgq* transcript served as an internal control to normalize the mRNA levels of the mutants to that of wild type. The *dgq* gene is predicted to make two transcripts (LEE *et al.* 1990), both of which appear in most lanes. Both transcripts, if present, were used in the quantitation. A blot from a separate gel (not shown) was probed in the same way with *ninaE* and *rp49* (O'CONNELL and ROSBASH 1984) sequences. The amounts of *ninaE* mRNA in mutant and wild-type flies, as determined from experiments using the *dgq* and *rp49* references, are shown under the autoradiograms.

have improved rhabdomere structure and more R1–6 cell activity than the $ninaE^{o117}$ null allele, we constructed two more *ninaE* mutants in order to understand the causes of the improved phenotypes. The first mutant, $ninaE^{205/309}$, has termination codons at sites 205 (UAA) and 309 (UGA). The second mutant, $ninaE^{\Delta 309}$, contains a deletion that removes all coding information downstream of the mutated 309 UGA termination codon, but leaves intact the endogenous termination codon and all downstream mRNA processing signals. Extra R1–6 activity resulting from translational (natural) readthrough at premature termination codons, thus producing a few full length opsins, would be deterred ($ninaE^{205/309}$) or prevented ($ninaE^{\Delta 309}$) in these mutants. However, any extra activity resulting from residual function of the truncated opsins would not be affected in these mutants. These mutant flies were backcrossed to the $ninaE^{o117}$ transformation stock for histological and electrophysiological analysis.

The analysis of these mutants indicates the truncated opsins are able to improve rhabdomere structure. $ninaE^{\Delta 309}$ (Figure 7B) has rhabdomeres even larger than those of $ninaE^{309UGA}$ flies (Figure 4D), and $ninaE^{205/309}$, as well, has a rhabdomere morphology comparable to $ninaE^{205UGA}$ (compare Figures 7A and 4C). Off-transient analysis (Figure 6D) shows the $ninaE^{\Delta 309}$ mutants have a reduction in R1–6 cell activity compared to any $ninaE^{309}$ nonsense allele, whereas the $ninaE^{205/309}$ mutants show no change compared to the $ninaE^{205}$ nonsense alleles. The activity level of $ninaE^{\Delta 309}$ is similar to that of $ninaE^{205/309}$ (Figure 6D) and of all $ninaE^{205}$ nonsense alleles; that is, they respond with off-transients to only the brightest light. Thus, the deletion eliminates the extra R1–6 rhodopsin activity seen in the $ninaE^{309}$ nonsense mutants, suggesting the extra rhodopsin activity observed in the ERG of these mutants arises from leaky termination at stop codon 309. As shown below, the basal activity of $ninaE^{205/309}$, $ninaE^{\Delta 309}$, and $ninaE^{205}$ alleles is also evident in $ninaE^{o117}$ flies upon outcrossing. As the truncated opsins maintain rhabdomere structure, but do not improve the physiology of the photoreceptor cells, the level of rhodopsin active in phototransduction is not correlated to rhabdomere structure in these mutants.

Genetic suppression of *ninaE* nonsense alleles: *ninaE* nonsense mutants were genetically suppressed by crossing flies homozygous for the mutant opsin gene to a stock with a second chromosome bearing a $tRNA^{Leu}$ nonsense suppressor [DtL^a Su^+ (opal), DtL^b Su^+ (opal), or DtL^b Su^+ (amber); GARZA, MEDHORA and HARTL (1990)] maintained over the SM1 balancer chromosome. Each stock contains a tRNA gene mutated at the anticodon so that leucine amino acids are inserted at UGA or UAG termination codons, respectively. These suppressor stocks were made by site-directed mutagenesis of cloned tRNA genes and subsequent transformation of flies (GARZA, MEDHORA and HARTL 1990). Figure 8 shows EM sections from genetically suppressed *ninaE* nonsense alleles (genotypes *w*; P[ry^+, DtL^a Su^+ (opal)]; P[ry^+, $ninaE^{205UGA}$], Figure 8A, and *w*; P[ry^+, DtL^a Su^+ (opal)]; P[ry^+, $ninaE^{309UGA}$], Figure 8B). The presence of a genetic suppressor in these flies does not change the morphology of the R1–6 rhabdomeres (compare to Figure 4, C and D, cross-sections from their unsuppressed siblings).

ERG phenotypes of genetically suppressed nonsense alleles: Figure 9, A and B, show off-transient analyses for the same opal alleles with and without the genetic suppressor DtL^a Su^+ (opal). The presence of the suppressor in each case increases the sensitivity of the outer photoreceptor cells so that the flies produce transients in response to lights dimmer than those

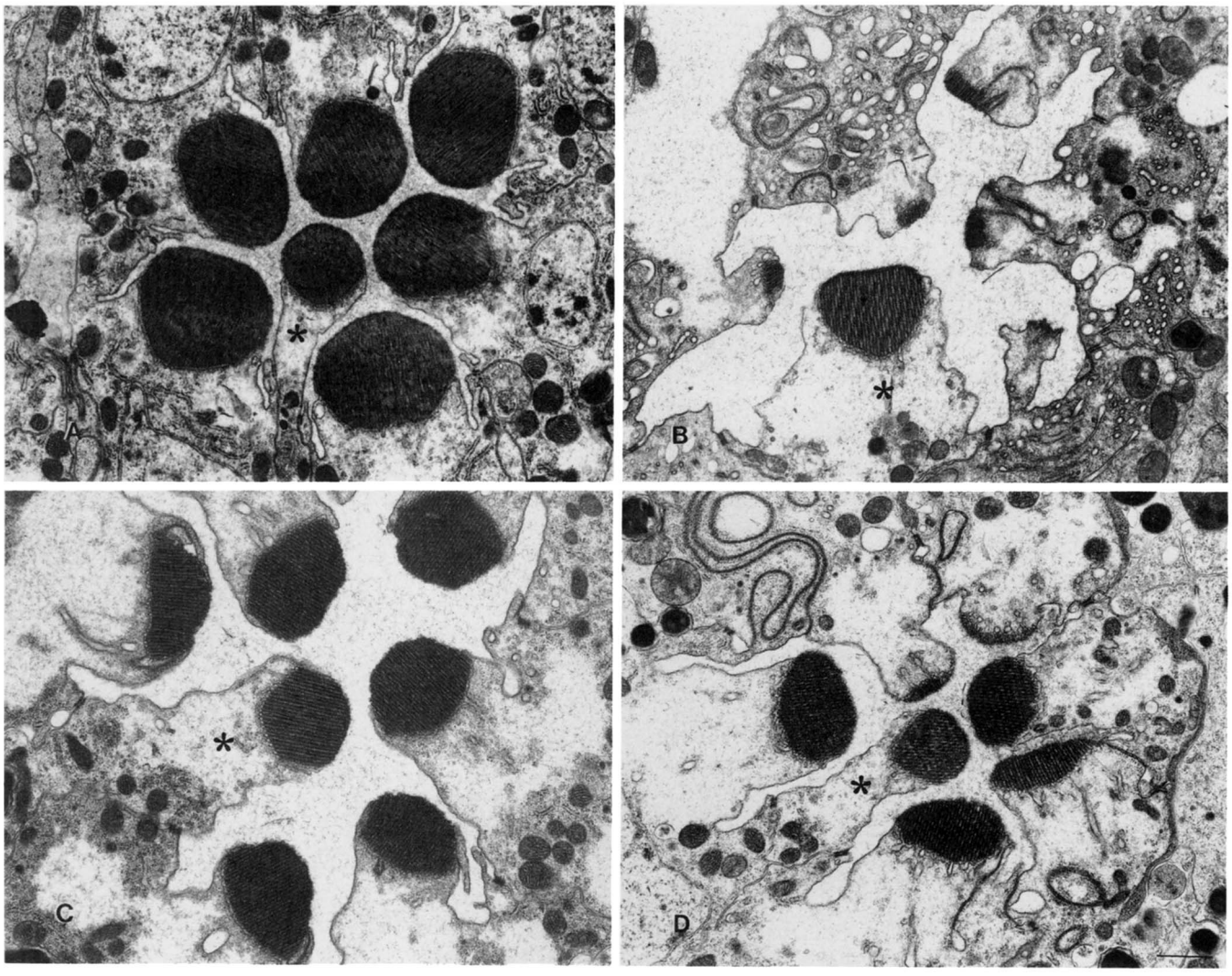


FIGURE 4.—Electron micrographs of wild-type and mutant ommatidial cross sections. Wild type (A), $ninaE^{o117}$ (B), $ninaE^{205UGA}$ (C), and $ninaE^{309UGA}$ (D) are shown. Sections were cut from the distal third of the retina; each ommatidium at this level contains the rhabdomere from the R7 photoreceptor cell, as indicated by an asterisk. All other rhabdomeres belong to the R1–6 class of photoreceptor cells. The flies in (c) and (d) are the siblings of the flies depicted in Figure 8. They carry the SM1 balancer chromosome and 1 copy of the *P* element insert. Each fly was aged 1.5–2.5 days posteclosion before fixing. The bar represents 1 μm.

needed for unsuppressed flies. In the absence of a genetic suppressor, $ninaE^{309UGA}$ has more electrophysiological activity than $ninaE^{205UGA}$, but the opposite is true in the presence of a suppressor. More $ninaE^{205UGA}$ flies (35%, Figure 9A) respond with transients to the −2 intensity than do $ninaE^{309UGA}$ flies (5%, Figure 9B).

The nonsense allele $ninaE^{205UAG}$, not expected to be suppressed by the opal genetic suppressor, shows no increase in R1–6 cell activity in siblings carrying the genetic suppressor over those carrying the balancer chromosome (Figure 9C). $ninaE^{o117}$, also not expected to be affected by the suppressor, shows similar results (Figure 9D). The $ninaE^{o117}$ siblings responded with transients to bright light with some frequency, regardless of whether they carried a genetic suppressor (Figure 9D; also see MATERIALS AND METHODS). Therefore, the suppression detected in crosses with opal alleles of *ninaE* is specific to readthrough at opal termination codons and is not a consequence of genetic background effects.

The increased R1–6 cell activity in the presence of the DtL^a opal suppressor as evident in the off-transient analysis was confirmed by ERG amplitude analysis. ERG amplitudes from suppressed and unsuppressed $ninaE^{205UGA}$ siblings and from progeny flies of a similar cross involving $ninaE^{P332}$ (a missense allele not expected to be suppressed by a nonsense suppressor) were compared. The brightest light produced an average ERG amplitude difference of 4.3 mV between the suppressed and unsuppressed siblings of the $ninaE^{205UGA}$ genotype (Figure 10); this difference was significant by Student's *t* test ($P = 0.0080$), whereas the difference between $ninaE^{P332}$ suppressed and unsuppressed siblings, 0.9 mV, was not. Additionally, the mean amplitude difference between $ninaE^{205UGA}$ and $ninaE^{P332}$ flies (0.2 mV), each carrying the non-

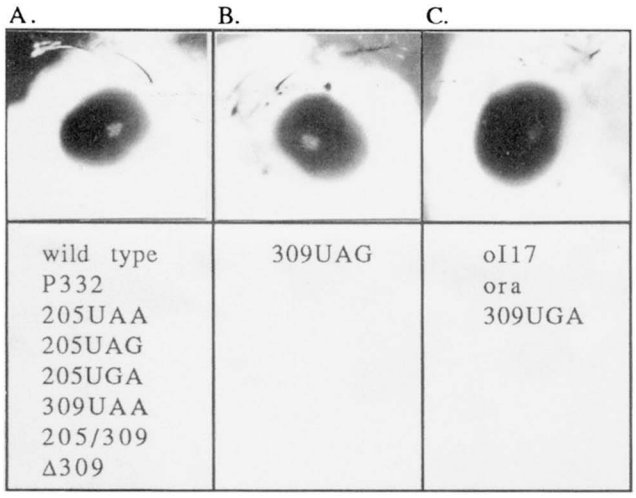


FIGURE 5.—Deep pseudopupils of wild-type and *ninaE* mutant flies. The pseudopupil appearance correlates well with the EM analysis of rhabdomere structure. The *ninaE* mutants were classified into three groups based on their pseudopupil appearance 1.5–2.5 days posteclosion. An example of each group is shown: (A) similar to wild type (all seven spots are bright and clear), (B) intermediate in phenotype (the central spot is brighter and more distinct than the outer ones), and (C) similar to $ninaE^{oI17}$ (only the central spot is visible). Each mutant was homozygous for the *P* element insert.

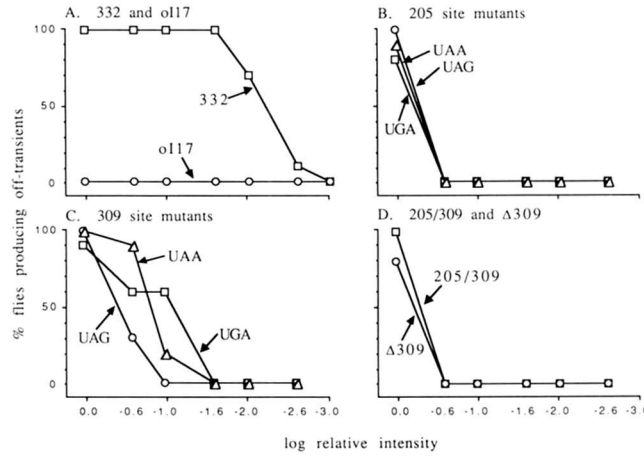


FIGURE 6.—Off-transient frequency of *ninaE* mutants. Each graph shows the percentage of flies that produced off-transients at each light intensity (for details of the procedure, see MATERIALS AND METHODS). The $ninaE^{oI17}$ (○) and $ninaE^{P332}$ (□) mutant alleles are shown in (A), the $ninaE^{205UAA}$ (△), $ninaE^{205UAG}$ (○), and $ninaE^{205UGA}$ (□) alleles in (B), the $ninaE^{309UAA}$ (△), $ninaE^{309UAG}$ (○), and $ninaE^{309UGA}$ (□) alleles in (C), and the $ninaE^{205/309}$ (□) and $ninaE^{\Delta 309}$ (○) alleles are shown in (D). $ninaE^{P332}$ and the site-directed mutants were progeny of a cross to the $ninaE^{oI17}$ injection stock; therefore, these flies carried one copy of the mutant *ninaE* gene in question. The flies were 1.5–2.5 days old, and 10 of each genotype were tested.

sense suppressor, was not significant, suggesting the R1–6 functional rhodopsin levels are similar in these flies.

Suppressors derived from the DtL^b gene are less efficient than the DtL^a derived opal suppressor. $ninaE^{205UGA}$ flies suppressed by the DtL^a-derived opal suppressor produced transients in response to lights no dimmer than the −1 intensity (Figure 9E). The amber suppressor derived from this tRNA gene is also a poor suppressor. $ninaE^{205UAG}$ flies suppressed with DtL^b Su^+ (amber) responded with transients to the −1 intensity, but not to dimmer lights (Figure 9F), and neither $ninaE^{309UAG}$ nor $ninaE^{ora}$ was affected by this amber suppressor (Figure 9, G and H).

DISCUSSION

The construction and analysis of nonsense mutations at two leucine sites within the major rhodopsin gene, *ninaE*, has enabled us to document genetic and natural nonsense suppression in Drosophila. The nonsense suppressors used in this study were constructed by GARZA, MEDHORA and HARTL (1990) by site-directed mutagenesis of cloned tRNA genes. These suppressors were then reintroduced into Drosophila strains by *P* element-mediated transformation.

Genetic suppression: Termination codons placed at leucine codon 205 in rhodopsin are more suppressible than those at the 309 site, as tested by DtL^a and DtL^b genetic suppressors. $ninaE^{205UGA}$ is suppressed to a greater extent than $ninaE^{309UGA}$ by DtL^a Su^+ (opal). The amber suppressor derived from the DtL^b gene weakly suppresses $ninaE^{205UAG}$ and does not detectably suppress $ninaE^{309UAG}$. This suppressor also does not suppress the $ninaE^{ora}$ amber mutation, but since this nonsense mutation occurs at a glutamine codon (WASHBURN and O'TOUSA 1989), it is possible that the insertion of leucine by DtL^b Su^+ (amber) results in a nonfunctional opsin. The detection of $ninaE^{ora}$ suppression may be limited, as well, by the reduced $ninaE^{ora}$ transcript level (10–20% of wild type). However, the $ninaE^{205}$ and $ninaE^{309}$ suppression efficiency differences cannot be attributed to *ninaE* mRNA availability, since the $ninaE^{309UGA}$ transcript is twice as abundant and less efficiently suppressed than $ninaE^{205UGA}$.

The efficiencies of nonsense suppressors derived from two Drosophila tRNA genes, DtL^a and DtL^b, were compared. The DtL^a derived opal suppressor is more efficient than the opal suppressor derived from DtL^b, as tested on $ninaE^{205UGA}$. It was previously suggested, as well, that the amber suppressor of DtL^a is stronger in flies than the amber suppressor of DtL^b (GARZA, MEDHORA and HARTL 1990). These differences must be due to differential tRNA gene expression or processing, since the tRNA gene products of DtL^a and DtL^b show differences only within an intron (ROBINSON and DAVIDSON 1981). However, the expression levels of these nonsense suppressors may not accurately reflect the endogenous expression of the $tRNA^{Leu}$ genes from which they were derived. Position effects, such as noted for a *C. elegans* tRNA nonsense suppressor (FIRE 1986), may alter the

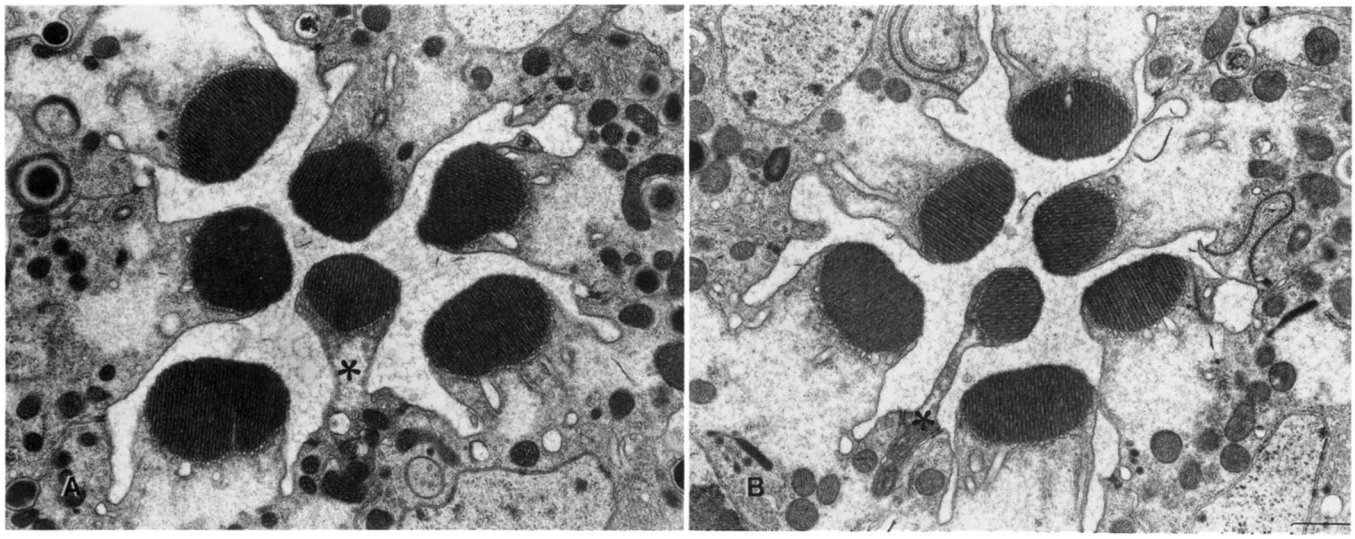


FIGURE 7.—Rhabdomere structure of $ninaE^{205/309}$ and $ninaE^{\Delta 309}$ mutants. Electron micrographs of cross-sections from $ninaE^{205/309}$ (A) and $ninaE^{\Delta 309}$ (B) ommatidia show the structure of the R1–6 rhabdomeres in these mutants. The R7 cells in these sections are indicated by asterisks. Each fly contains one copy of the mutant gene indicated, as these are progeny from crosses to $ninaE^{ol17}$ flies. Each fly was aged between 1.5 and 2.5 days posteclosion. The bar represents 1 μm.

expression level of the transformed tRNA genes.

Drosophila genetic suppression efficiencies assayed in previous studies are less than 1% (DOERIG *et al.* 1988; GARZA, MEDHORA and HARTL 1990; LASKI *et al.* 1989). GARZA, MEDHORA and HARTL (1990) showed that DtL^b Su^+ (amber) suppressed an amber mutation in the bacterial chloramphenicol acetyl transferase gene with an efficiency of 0.4%. We have shown that the same tRNA suppressor suppressed the $ninaE^{205UAG}$ allele with an efficiency estimated to be less than 0.04%. This value is based on a comparison of $ninaE^{P332}$ and $ninaE^{205UAG}$ (Su^+) functional rhodopsin levels; $ninaE^{P332}$ flies have 0.04% rhodopsin activity and $ninaE^{205UAG}$ (Su^+) flies have even less. The difference in suppression efficiencies may be due to codon context effects (for a review, see VALLE and MORCH 1988) or perhaps to a lower expression level of the tRNA gene in photoreceptors relative to other tissues of the fly. There is precedent for the latter, as tissue specific expression of tRNA genes has been noted in silkworms (SPRAGUE, HAGENBUCHLE and ZUNIGA 1977), and a survey of nonsense suppressor efficiencies in *C. elegans* has shown that some tRNA suppressors are more efficient in one tissue than others (HODGKIN 1985; KONDO, HODGKIN and WATERSTON 1988; KONDO *et al.* 1990). Taken together, all Drosophila studies have indicated that suppression rates for existing Drosophila nonsense suppressors are lower than seen in other experimental systems, suggesting that tRNA-mediated genetic suppression is an

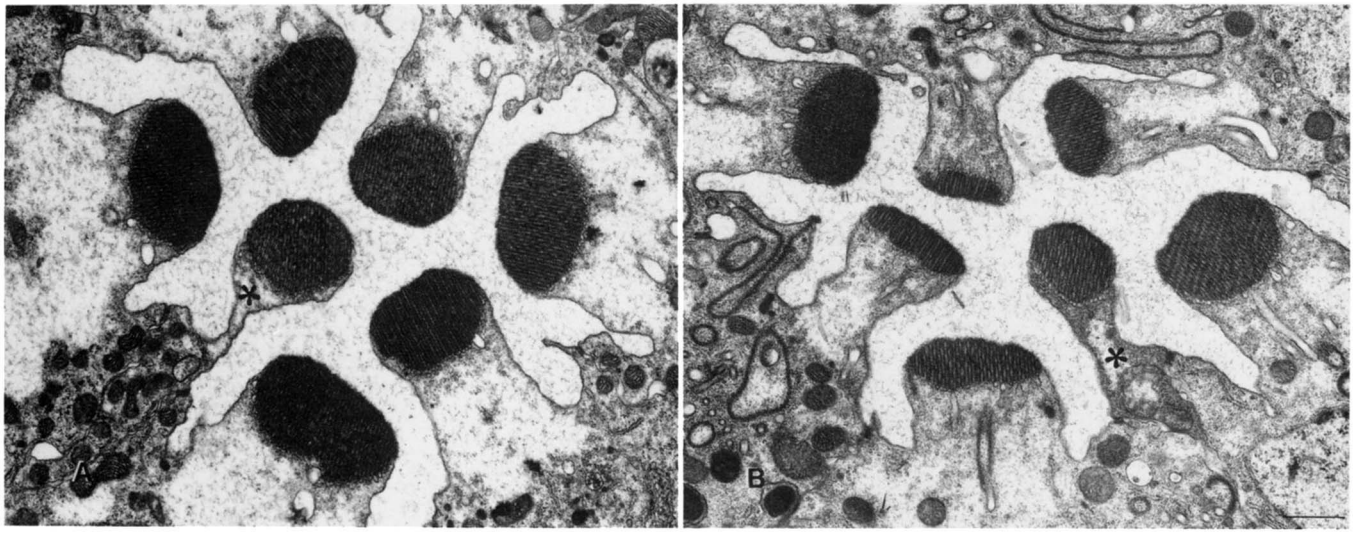


FIGURE 8.—Ommatidial cross sections of genetically suppressed flies. $ninaE^{205UGA}$ (A) and $ninaE^{309UGA}$ (B) flies carry one copy of the *ninaE* nonsense mutation and one copy of the tRNA suppressor DtL^a Su^+ (opal). The R7 cell is indicated by an asterisk in each micrograph. Flies were aged 1.5–2.5 days posteclosion. The bar represents 1 μm.

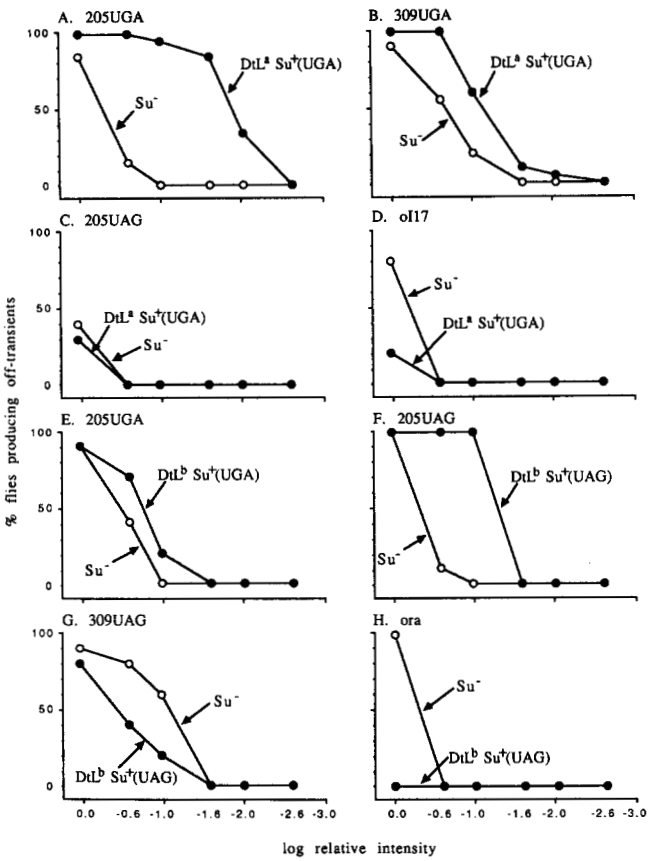


FIGURE 9.—Off-transient frequency of genetically suppressed nonsense mutants. The percentage of flies that produced off-transients at each light intensity is depicted (for details of the procedure, see MATERIALS AND METHODS). Sibling flies carried either the tRNA suppressor *DtL^a Su$^+$* (opal) or the SM1 balancer chromosome (*Su$^-$*) in combination with *ninaE^{205UGA}* (A), *ninaE^{309UGA}* (B), *ninaE^{205UAG}* (C), or *ninaE^{oI17}* (D). Neither *ninaE^{205UAG}* nor *ninaE^{oI17}* is specifically suppressed by the opal suppressor, so these serve as negative controls. Sibling flies carried either the tRNA suppressor *DtL^b Su$^+$* (opal) or the SM1 balancer chromosome (Su$^-$) in combination with *ninaE^{205UGA}* (E), and either the tRNA suppressor *DtL^b Su$^+$* (amber) or the SM1 balancer chromosome (Su$^-$) in combination with *ninaE^{205UAG}* (F), *ninaE^{309UAG}* (G), and *ninaE^{ora}* (H). Twenty flies of each genotype were assayed in (A) and (B), and 10 flies were assayed for all other genotypes.

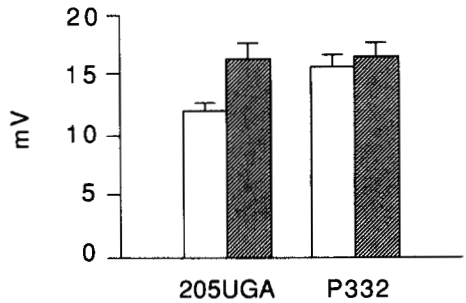


FIGURE 10.—ERG amplitudes of genetically suppressed flies. *ninaE^{205UGA}* and *ninaE^{P332}* flies were crossed to the *DtL^a Su$^+$* (opal) suppressor stock such that their progeny carry either the tRNA suppressor (hatched boxes) or the SM1 balancer chromosome (*Su$^-$*) (open boxes). The missense mutant, *ninaE^{P332}*, is not predicted to be suppressed by a nonsense suppressor and serves as a negative control. The mean amplitude of the ERGs taken from these flies in response to bright white light (1000 foot-candles) is presented and marked with standard error bars. The difference between the suppressed and unsuppressed *ninaE^{205UGA}* siblings is significant by Student's *t* test ($P = 0.008$). The difference between suppressed and unsuppressed *ninaE^{P332}* is not significant ($P = 0.575$) and the difference between *ninaE^{205UGA}* and *ninaE^{P332}*, both of which carried the genetic suppressor, is also not significantly different ($P = 0.9233$). 10 flies of suppressed and 10 of unsuppressed *ninaE^{P332}* were tested, while 15 flies of suppressed and 16 flies of unsuppressed *ninaE^{205UGA}* were tested.

inefficient process in Drosophila. However, it may be that none of the cloned genes used to make suppressors are capable of mutating to strong suppressors or that position effects diminish their expression and hence their suppression potential.

Natural suppression: Flies carrying *ninaE^{309}* nonsense alleles have more rhodopsin activity than the *ninaE^{oI17}* null mutant or flies carrying the *ninaE^{205}* or *ninaE^{ora}* nonsense alleles. To determine whether the residual activity was due to the function of the truncated product, the *ninaE^{309}* gene was deleted at its 3′ end just downstream of the 309 nonsense mutation (*ninaE$^{\Delta 309}$*). In this deletion mutant, the photoreceptor activity is reduced to basal levels. Thus, the truncated opsin (308 amino acids long) does not initiate phototransduction. The activity seen in the 309 nonsense mutants must result from natural readthrough at the 309 stop codon. Therefore, in these mutants, even in the absence of a genetic suppressor, an amino acid is incorrectly inserted at the stop codon to produce a small amount of full length, functional opsin.

Natural suppression occurs at all three termination codons at the 309 site. Therefore, endogenous tRNAs are able to read (or misread) UAA, UAG or UGA at this site. Readthrough at all three termination codons has been recently reported for transcripts of eukaryotic viruses, as well (FENG *et al.* 1989; LI and RICE 1989). UAA has been speculated to be unique among the three termination codons in preventing natural readthrough in eukaryotes (GELLER and RICH 1980). However, in our experiments, UAA is suppressed at a similar level as UAG and UGA.

The efficiency of natural readthrough and genetic nonsense suppression at the 205 and 309 sites is likely modulated by tRNA-specific context effects. The three nonsense mutations are naturally suppressed at the 309 site, but not detectably at the 205 site. We suggest that the observed differences between natural suppression at the 205 and 309 sites are likely due to context effects modulating the efficiency of readthrough. Another explanation, that the insertion of an amino acid other than leucine at site 205 produces a nonfunctional opsin, is not supported by our results. GELLER and RICH (1980) observed that the the endogenous UGA-suppressing tRNA in a reticulocyte assay is likely mediated by a tryptophan-inserting tRNA. We constructed and tested missense mutants that place tryptophan at either site 205 or 309 and

found that both mutants produce at least 20% functional rhodopsin (data not shown). Thus, the occurrence of natural suppression, at least as mediated by $tRNA^{Trp}$, would be detectable at both sites. Although the 205 site does not show natural suppression, it is more suppressible than the 309 site with either DtL^a or DtL^b genetic suppressors. Since tRNAs respond differently to codon contexts, tRNA-specific factors likely regulate the efficiency of the suppression. Such tRNA-specific context effects have been described in bacteria, and may result from tRNA-tRNA interactions at the A and P sites on the ribosome (BOSSI 1983; BOSSI and ROTH 1980; MILLER and ALBERTINI 1983; SMITH and YARUS 1989).

Rhodopsin and rhabdomere structure: Expression of the opsin protein is essential for the structure of rhabdomeres, as $ninaE^{o117}$, a null allele, produces vestigial rhabdomeres that degenerate within a few days of eclosion (O'TOUSA, LEONARD and PAK 1989). On the other hand, missense alleles making only 0.1% active rhodopsin maintain nearly normal rhabdomeres (O'TOUSA, LEONARD and PAK 1989). We expected that the rhabdomere morphology would be a convenient assay for selecting or examining nonsense suppressors. However, we have shown that the full length opsin protein is not a requirement for rhabdomere maintenance. Expression of the truncated opsins produced from the $ninaE^{205/309}$ and $ninaE^{\Delta 309}$ mutants maintains rhabdomere structure, and as a result, rhabdomere morphology could not be used as a phenotype to document genetic suppression of these *ninaE* nonsense mutants.

Though the rhabdomere phenotype has not been useful in documenting genetic suppression, it has been useful in defining a few parameters of rhodopsin-mediated rhabdomere maintenance. These truncated proteins lack the C-terminal half ($ninaE^{205}$) or fourth ($ninaE^{309}$) of the opsin and are still able to maintain rhabdomere structure. The truncated opsins do not possess the retinal binding site, but contain other important residues, such as the N-terminal glycosylation site (O'TOUSA 1992) and the cysteines involved in disulfide bridge formation (KARNIK and KHORANA 1990; KARNIK *et al.* 1988), necessary for the processing and transport of rhodopsin. The 204 amino acid opsin of $ninaE^{205UGA}$, approximately half the length of the wild-type opsin, maintains rhabdomere structure better than the 308 amino acid protein of $ninaE^{309UGA}$. It is not likely that this results from increased *ninaE* expression in the $ninaE^{205UGA}$ transformant, because the $ninaE^{309UGA}$ mutant has approximately twice the steady state *ninaE* mRNA of the $ninaE^{205UGA}$ mutant. These results suggest that truncated protein length is not as critical as other protein properties, such as stability or structure, for the maintenance of rhabdomeric membranes.

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