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Headless flies produced by mutations in the paralogous *Pax6* genes *eyeless* and *twin of eyeless*

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SUMMARY

The two *Pax6* gene homologs *eyeless* and *twin of eyeless* play decisive early roles in *Drosophila* eye development. Strong mutants of *twin of eyeless* or of *eyeless* are headless, which suggests that they are required for the development of all structures derived from eye-antennal discs. The activity of these genes is crucial at the very beginning of eye-antennal development in the primordia of eye-antennal discs when *eyeless* is first activated by the *twin of eyeless* gene product. This activation does not strictly depend on the Twin of eyeless protein, but is temperature-dependent in its absence. Twin of eyeless acts also in parallel to the *eyeless* gene and exerts functions that are partially redundant with those of Eyeless, while Eyeless is mainly required to prevent early cell death and promote eye development in eye-antennal discs.

Key words: Headless flies, *Pax6* genes, *twin of eyeless*, *eyeless*, Head development, Eye development, *Drosophila melanogaster*

INTRODUCTION

Pax genes, discovered by a test of the gene network hypothesis (Bopp et al., 1986; Frigerio et al., 1986), play crucial roles in the development of the central nervous system (CNS) and brain, as well as of the peripheral nervous system and sensory organs (Noll, 1993; Dahl et al., 1997). They encode transcription factors that bind to specific DNA sequences by virtue of a conserved paired-domain and have been divided into four different classes according to the four primordial *Pax* genes present at the time of protostome-deuterostome separation (Noll, 1993). Two classes include paired-domain proteins with a second DNA-binding domain, a *prd*-type homeodomain. *Pax* genes belonging to the same class frequently overlap in their spatiotemporal expression patterns because the product of one gene activates transcription of the other (Noll, 1993). In contrast, paired-domain proteins of separate classes avoid such overlaps, presumably because they activate interfering developmental pathways (Jiao et al., 2001). In *Drosophila*, for example, the product of the pair-rule gene *paired* activates the segment-polarity gene *gooseberry* in the epidermis (Li et al., 1993), whose product in turn activates *gooseberry-neuro* in the neuroectoderm (Gutjahr et al., 1993). All three genes belong to the same class as the mammalian *Pax3/Pax7* genes. Similarly, the four *Drosophila* genes *eyeless* (*ey*), *twin of eyeless* (*toy*), *eye gone* (*eyg*), and *twin of eyg* (*toe*) that belong to the vertebrate *Pax6* class are expressed in the same developmental pathway controlling eye development (Quiring et al., 1994; Jun et al., 1998; Czerny et al., 1999; Jang and Sun, 2001). Also in this case, it has been proposed that the Toy protein is required for the activation of the *ey* gene, both of which were thought to be required for proper development of the eye disc, yet not of the antennal disc (Czerny et al., 1999). In the absence of *toy* mutants, however, this proposal was based entirely on experiments testing for ectopic activation of *ey* upon ectopic expression of *toy*.

In order to clarify the roles of the two *Pax* genes *ey* and *toy* in the development of normal rather than ectopic eyes, we examined the effect on eye development of the first *toy* mutations and of a much stronger *ey* allele than previously characterized. We concentrated particularly on the earliest period of *ey* and *toy* expression in the eye-antennal primordia in embryos (Czerny et al., 1999). This period has been shown to be particularly sensitive to developmental pathway interference, which generates headless flies (Jiao et al., 2001), a phenotype much stronger than that observed for *ey* mutants (Quiring et al., 1994; Halder et al., 1998). As this indicated much wider roles for *toy* and *ey* in head development, we set out to find strong mutants of both *toy* and *ey* in order to examine if these displayed a headless rather than the milder eyeless phenotype.

We found that strong mutants of either *toy* or *ey* indeed lack all structures derived from eye-antennal discs and thus exhibit a spectacular headless phenotype. In addition, activation of *ey* in the primordia of eye-antennal discs does not strictly depend on the presence of the product of the *toy* gene, but becomes

temperature-sensitive in its absence. In the absence of Toy protein, moderate levels of Ey protein are sufficient to rescue the headless phenotype, while high Ey levels are necessary to rescue the eyeless phenotype as well. In contrast, very low levels of Ey apparently suffice to rescue the headless phenotype in the presence of wild-type Toy protein. These findings suggest a delicate balance of Ey protein levels regulating eye-antennal disc development and a partial redundancy of Ey and Toy functions in these discs, in which Toy acts not only through Ey, but also in a pathway parallel to Ey. Finally, inhibition of apoptosis by the baculovirus P35 protein is able, in the absence of functional Ey, to rescue the headless, but not the eyeless phenotype. Hence, Ey is not responsible for the development of head structures derived from the antennal disc, but is primarily required to inhibit cell death and to promote eye development.

MATERIALS AND METHODS

General procedures and fly stocks

The preparation of genomic libraries, isolation of poly(A)$^+$ RNAs or cDNAs, 5′- and 3′-RACE of mRNAs, northern blot analysis, PCR, DNA sequencing, and in situ hybridization to salivary gland chromosomes were carried out according to standard procedures. Mutant stocks not obtained from the Umea and Bloomington Stock Centers have been described previously (Fu et al., 1998; Jiao et al., 2001) or are characterized here.

Determination of the lethal phase of *toy^{hdl}* mutants

To assay the lethal phase of homozygous *toy^{hdl}* mutants, newly hatched first instar larvae were picked from a *toy^{hdl}/spa^{Cat}* stock and the fractions that reached the pupal and adult stages determined. These fractions were the same at all temperatures tested (18, 23, 25, and 28°C). All viable adults were heterozygotes because all *spa^{Cat}* homozygotes die during embryogenesis (Hochman, 1976). From a total of 3250 larvae, 2728 developed to pupae and 1853 to viable adults. Assuming that of the 15% of heterozygotes that fail to reach the adult stage half each die as larvae or pupae, we find that the lethality of *toy^{hdl}* mutants is 34±2% during the larval and 66±3% during the pupal stage.

In situ hybridization of RNA probes to embryos

In situ hybridization with antisense RNA probes to staged embryos was carried out according to standard procedures. To determine what fraction of *toy^{hdl}* mutants showed a reduced level of *ey* transcripts in the eye-antennal primordia, their embryonic stage was determined and the level of *ey* transcripts compared to that of late wild-type embryos of the same stage. Among embryos derived from *toy^{hdl}/l(4)2C2* parents, the level was reduced in 32 out of 127 late stage 16 embryos at 25°C, which is as expected if it was reduced in all homozygous *toy^{hdl}* embryos; and in 10 out of 124 such embryos at 18°C, i.e., in a third of all *toy^{hdl}* embryos (Fig. 5A,C,E). For embryos derived from *Df(4)spa^{66}/l(4)2C2* parents, it was first verified that 25% of all stage 16 embryos were homozygous for the deficiency, by the absence of *toy* transcripts after in situ hybridization with a *toy* cDNA probe, before the same analysis for *ey* transcript levels was carried out. Again the level was reduced in a quarter (24/100) of late stage 16 embryos at 25°C, and in about half (10/71) of the expected number of homozygous *Df(4)spa^{66}* embryos of the same stage at 18°C (Fig. 5G,H). Similar results were obtained for embryos from *Df(4)spa^{30}/l(4)2C2* parents: *ey* transcripts in the eye-antennal primordia were clearly reduced in 17 out of 62 embryos at 25°C, while they were reduced in only 13 out of 100 embryos at 18°C.

RESULTS

The *l(4)8* mutation generates headless flies

Both *Drosophila* homologs of the vertebrate *Pax6* gene, *ey* and its paralog *toy*, are located on the small fourth chromosome (Czerny et al., 1999). No mutants of *toy* have been described, while only hypomorphic *ey* alleles have been identified, the strongest of which give rise to eyeless adult flies (Quiring et al., 1994; Halder et al., 1998). This is surprising since the fourth chromosome has been subjected to an extensive systematic screen for lethal mutations in an attempt to establish its total number of such loci (Hochman et al., 1964; Hochman, 1971; Hochman, 1976). However, a change in nomenclature of the mutant alleles, by which new alleles (Hochman, 1971; Hochman, 1976) were given names of alleles found in a previous screen (Hochman et al., 1964), caused considerable confusion, and many of these mutants were lost. In May 1994, in the course of examining the phenotype of some of these mutant stocks more closely, one of us (Å. R.-L.) discovered that homozygous *l(4)8* flies (Hochman et al., 1964), which die as pharate adults inside their pupal case, have a spectacular phenotype. When raised at 28°C, 65% of these pharates were headless, developing no structures derived from the eye-antennal discs (Fig. 1A,B), while less extreme phenotypes had partially developed heads like ‘half-heads’ (Fig. 1C) and ‘cleft-heads’ (Fig. 1D). The weakest phenotype was a ‘small-eye’ (Fig. 1E), similar to heterozygous *ey^D* flies, or it was indistinguishable from wild type (Fig. 1F). According to its most extreme phenotype, the *l(4)8* mutant allele was called *headless* (*hdl*).

The headless phenotype of *hdl* mutants is temperature-sensitive with a phenocritical period at the onset of *ey* transcription in the anlagen of the eye-antennal discs

The relative proportions of strong, intermediate and weak phenotypes observed among homozygous *hdl* flies depend on the temperature at which they are raised. Thus, at 18°C about 3% of these flies survived to apparently wild-type fertile adults, while 65% of the pharates had normal heads and only about 3% were headless. The remaining pharates developed partial heads and consisted of about equal portions of half-heads (Fig. 1C), cleft-heads (Fig. 1D) and small-eyed flies (Fig. 1E). In addition, about 1% each of the flies died as pharates in which only one eye and nearly no other head structures developed (one-eyed pharates) or as headless pharates with an eye inside their thorax (eye-in-thorax pharates). In contrast, at 28°C no *hdl* flies survived to viable adults, 65% of the pharate adults were headless (Fig. 1A,B), and only about 10% exhibited an apparently wild-type head phenotype. The remaining 25% of the pharates consisted of about equal portions of eye-in-thorax, one-eyed, and half-head flies, while only about 1% each of the pharates were small-eyed or cleft-heads.

This strong temperature sensitivity of the *hdl* allele permits us to test whether its temperature-sensitive functions are restricted to a critical period or required throughout development. To this end, temperature-shift experiments were carried out during which embryos developing at 19.5°C were transferred to 28°C (shift-up experiments; Fig. 2A) or embryos developing at 28°C were shifted to 19.5°C (shift-down experiments; Fig. 2B) after various time intervals. In both

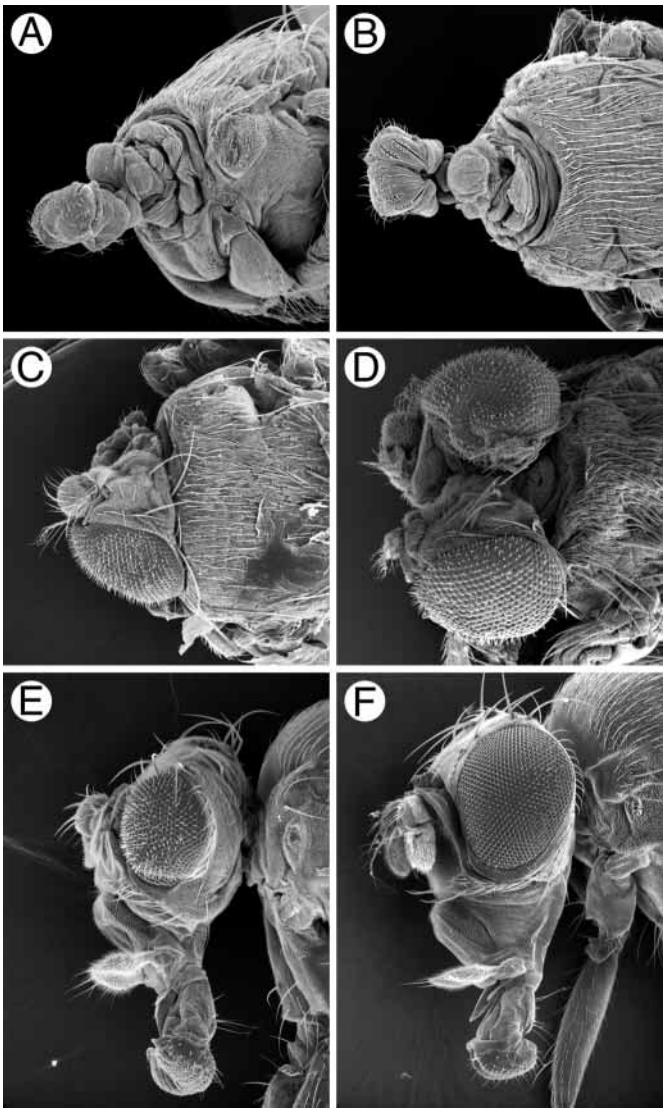

Fig. 1. Homozygous *l(4)8* flies show a headless phenotype. Scanning electron micrographs of the anterior portion of homozygous *l(4)8* pharate (A-D) or rare viable (E) adults and of a wild-type fly (F) are shown. The strongest phenotypes (A,B) are headless and lack all structures derived from the eye-antennal discs. Typical weaker phenotypes include half-heads (C) and cleft-heads (D). (A,E,F) Lateral, (B-D) dorsal views, anterior is to the left.

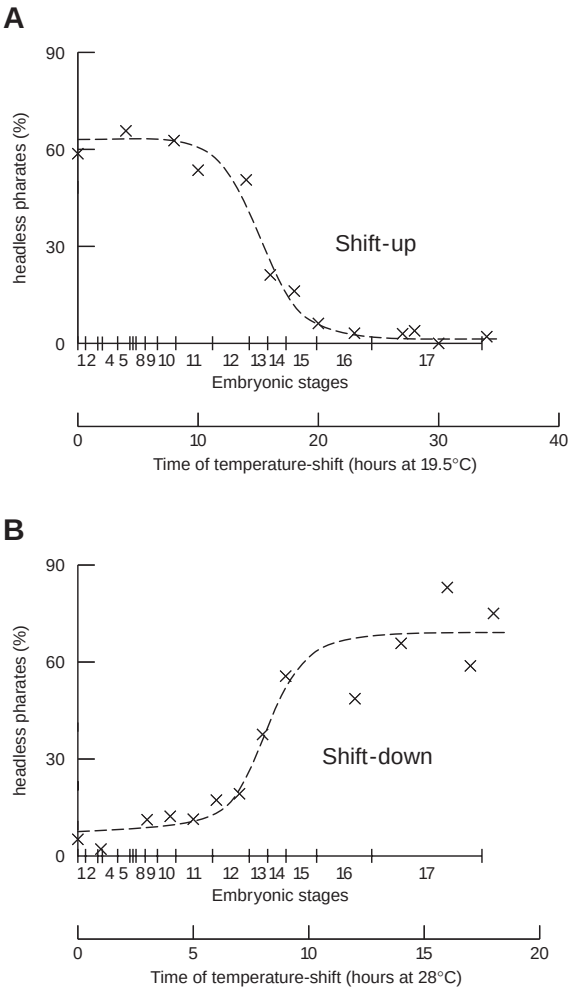

Fig. 2. Temperature-sensitive period of *hdl* mutant producing headless pharate adults. Embryos derived from a *hdl*/*ci*^{D} stock were collected for 4 hours and raised at 19.5°C (A), or collected for 2 hours and raised at 28°C (B), until the temperature was shifted to 28°C and 19.5°C, respectively, during embryonic development at the times indicated on the abscissa. The fraction of headless phenotypes among pharate adults is plotted against the time corresponding to the average age of the embryos at the time of the temperature shift. Points on the ordinate in A and B represent the fractions of headless pharates observed when the temperature was kept constant at 28°C (A) or 19.5°C (B) throughout development. Note that in both shift-up and shift-down experiments the temperature-sensitive period ends at stage 16 and begins at stage 12, the time when *toy* and *ey* transcripts begin to appear in the anlagen of the eye-antennal discs and in the optic lobe (Czerny et al., 1999). Embryonic stages are numbered according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).

temperature-shift experiments, the temperature-sensitive period was limited to a short interval from stage 12 to 16 (Campos-Ortega and Hartenstein, 1997), when *toy* and *ey* transcripts begin to appear in the anlagen of the eye-antennal discs (Jürgens and Hartenstein, 1993; Czerny et al., 1999). Temperature shifts after this phenocritical period showed the same proportions of headless phenotypes as if no shift had occurred.

In addition, about one third of the *hdl* homozygotes die as larvae (compare Materials and Methods), in agreement with the observed lethal phase of this mutant (Hochman, 1976). In contrast to the headless phenotype, larval lethality is independent of temperature between 18°C and 28°C. Thus, the *hdl* allele affects not only functions crucial for the proper development of the eye-antennal disc, but also additional vital functions required for survival to the pupal stage.

hdl is the first mutant allele of *toy*

Headless flies have been observed previously when ectopic expression of transcription factors in the eye-antennal primordia interfered with the developmental program initiated by the *toy* and/or *ey* genes (Jiao et al., 2001), both of which are located on the fourth chromosome (Czerny et al., 1999).

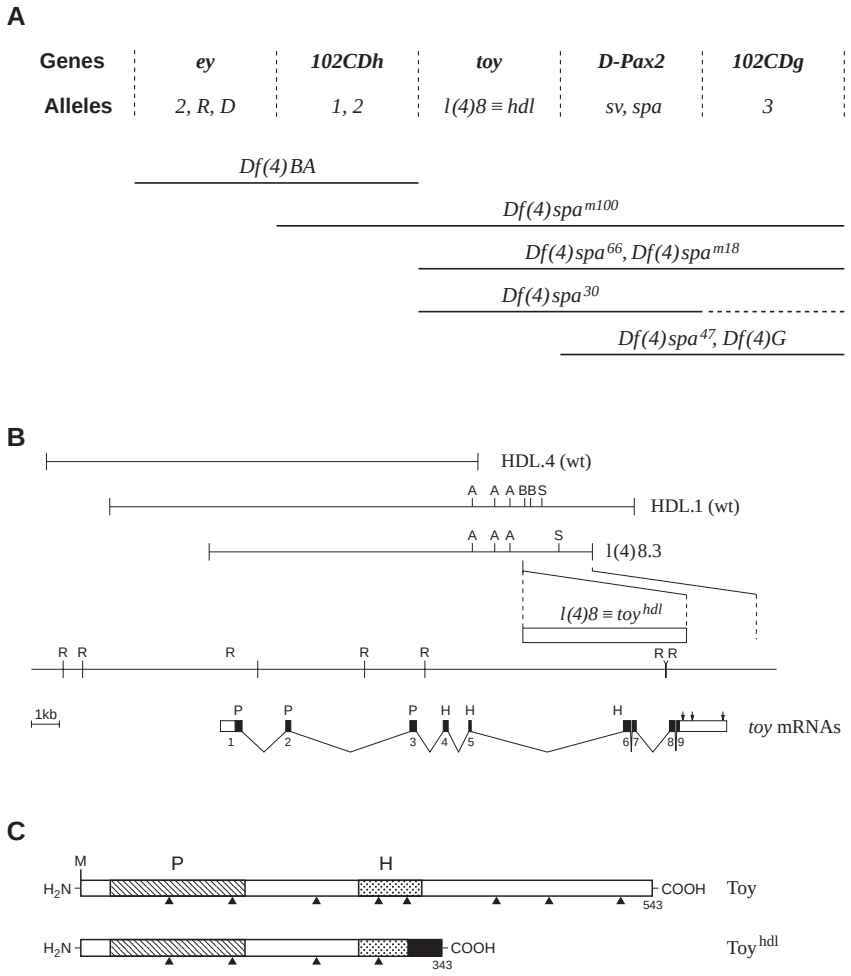

Fig. 3. The *hdl* allele is the first mutant allele of *toy*. (A) *hdl* is not allelic to *ey*, but located between *ey* and *D-Pax2*. A complete complementation analysis was carried out among the mutant alleles indicated below the loci of *ey*, *102CDh*, *toy*, *D-Pax2*, *102CDg*, and the deficiencies; the extent of each deficiency is indicated by horizontal lines below these loci. The *hdl* allele complements, and maps distal to, two mutant alleles of the *l(4)102CDh* locus, which are also located between *ey* and *D-Pax2* and one of which bears a synonym identical to that of an allele of the *l(4)8* locus according to the original nomenclature (Hochman et al., 1964). *hdl* also complements the *l(4)102CDg3* allele whose previous synonymous name, *l(4)19*, is identical to another allele of the *l(4)8* locus according to the original nomenclature. Since we have mapped *l(4)102CDg3* to the most distal portion of the right arm of chromosome 4, it is probably identical to the *l(4)19* mutation of the revised nomenclature, which has been mapped to this region (Hochman, 1971). The broken line of $Df(4)spa^{30}$ indicates incomplete complementation with the *102CDg* locus. The deficiencies $Df(4)spa^{30}$, $Df(4)spa^{47}$, $Df(4)spa^{66}$ (Fu et al., 1998), $Df(4)spa^{m18}$ and $Df(4)spa^{m100}$ have been obtained in two EMS-induced mutagenesis screens for *D-Pax2* mutants, while *Df(4)BA* uncovering *ey* was a gift from K. Basler (Brunner, 1997). The inverse sequence of the three loci *toy*, *D-Pax2* and *102CDg* in the right telomeric region of chromosome 4 is excluded because *Df(4)G*, which complements both *ey* and *hdl* but uncovers *D-Pax2*, is a telomeric deficiency (Hochman, 1971). The map is consistent with a previously published map (Locke et al., 2000), but not with that currently available at FlyBase, which erroneously localizes *toy* distal to *D-Pax2*. The map is further consistent with the gene order as determined by in situ hybridization to polytene chromosomes (Fu and Noll, 1997; Czerny et al., 1999). (B) The *hdl* mutation is a deletion of the 3′ portion of the *toy* transcript. The extent of genomic fragments isolated as clones from a wild-type (HDL.1 and HDL.4) and a homozygous *hdl* genomic library (l(4)8.3) in λ DASH II are shown with respect to an *Eco*RI restriction map below, derived from the genomic sequence provided by FlyBase. The genomic region deleted by the toy^{hdl} mutation is indicated above the restriction map and includes 5,863 bp, extending from nucleotide 1,855 of intron 5 to nucleotide 356 of the last exon, that are replaced by the five base pairs 5′-ATATC-3′. The exon-intron map shown below the genomic restriction map was determined by comparison of the genomic sequence with those of several *toy* cDNAs, isolated from an embryonic and an eye-disc cDNA library, and of products of a 5′-RACE with poly(A)$^+$ RNA from 0- to 20-hour-old embryos raised at 25°C. Protein coding portions of the exons are indicated in black, untranslated leader and trailer in white. Vertical arrows mark alternative 3′ ends as determined by sequencing of *toy* cDNAs and 3′-RACE products. They are preceded by a canonical poly(A) addition signal AATAAA with the exception of the first poly(A) addition site, which is preceded by CATAAA. Restriction sites: A, *Acc*I; B, *Bam*HI; R, *Eco*RI; S, *Sal*I. (C) The toy^{hdl} deficiency produces a truncated Toy^{hdl} protein. The wild-type Toy protein of 543 amino acids, including a paired-domain P and *prd*-type homeodomain H (Czerny et al., 1999), is shown schematically above the truncated Toy^{hdl} protein generated by the toy^{hdl} deficiency. The truncated protein consists of 343 amino acids and includes the N-terminal paired-domain, 46 amino acids of the homeodomain, and, if intron 5 is not spliced out, a 33 amino acid C-terminal portion encoded by the 5′ end of intron 5 whose first amino acid, Val, is identical to the 47th amino acid of the homeodomain. If intron 5 sequences are removed by splicing to a cryptic 3′ acceptor site close to the toy^{hdl} deficiency breakpoint in exon 9, the C-terminal tail of the truncated Toy^{hdl} protein (black) is shorter. The positions of introns are indicated by arrowheads.

Because *hdl* also maps to the fourth chromosome, we suspected that it is either the first mutant allele of *toy* or an allele of *ey* that is stronger than any of the previously characterized *ey* alleles. To test this hypothesis, we mapped *hdl* by complementation analysis, using a number of deficiencies that had been isolated in screens for *D-Pax2* mutants (Fu et al., 1998), a deficiency uncovering the *ey* locus (a generous gift from K. Basler) (Brunner, 1997), three *ey* alleles, and a few additional mutant alleles thought to belong to the same complementation group as *ey* or *hdl* on the basis of Hochman's complementation analysis (Hochman, 1971). The resulting genetic map shows that the *hdl* mutation is located distal to *ey* and proximal to *D-Pax2* (Fig. 3A). Therefore, *hdl* is not an *ey* allele, but its genetic location is consistent with the possibility that it is allelic to *toy*. To test this possibility, we isolated genomic clones of *toy* from wild-type and *hdl* λ phage libraries. Comparison of their restriction maps indeed suggested that sequences downstream of exon 5 of *toy* are deleted in *hdl* mutants (Fig. 3B). Isolation of the deficiency by PCR and subsequent DNA sequence analysis corroborated this

conclusion and demonstrated that *hdl* is a 5,863 bp deletion of the 3′ moiety of the *toy* transcript (Fig. 3B). It follows that *hdl* is the first mutant allele of *toy* and hence was named *toy^{hdl}*. Our finding is further consistent with a recent report, mentioning a headless phenotype of *toy* mutants as unpublished results (Kammermeier et al., 2001).

The *toy^{hdl}* deficiency produces a paired-domain protein truncated in the homeodomain

A developmental profile of *toy* transcripts obtained by northern blot analysis shows a strong and a weak mRNA band at about 3.8 kb and 2.7 kb, respectively, that are present throughout development as well as in adult flies (Fig. 4A). In addition, another weak band of 2.4 kb becomes visible in third instar larvae and continues to be expressed in subsequent stages. Since no maternal *toy* transcripts are detected, all bands are of zygotic origin. Their sizes are consistent with the mRNA lengths derived from *toy* cDNAs, extended by 5′- and 3′-RACE (Fig. 3B). Thus, the major *toy* mRNA includes a rather long 1.6 kb untranslated trailer sequence, while the minor bands reflect much shorter trailers (Fig. 3B). Analysis of *toy* mRNAs produced in homozygous or heterozygous *toy^{hdl}* flies reveals relatively strong bands at 4.7 kb and 2.8 kb, and weaker bands at 3.6 kb and 1.7 kb (Fig. 4B). The lengths of these new mRNAs indicate that the stronger bands correspond to the major 3.8 kb, the weaker ones to the minor 2.7 kb wild-type mRNA, if one assumes that intron 5 was either not removed or removed by splicing to a cryptic 3′ acceptor site close to the deficiency breakpoint in exon 9 (Fig. 3B). No change in *ey* mRNAs is observed in *toy^{hdl}* mutants, which confirms that the *toy^{hdl}* chromosome is wild-type at the *ey* locus (Fig. 4).

The observation that the levels of wild-type and *toy^{hdl}* mRNAs are about the same in adults (Fig. 4B) suggests that the deficiency does not remove a major enhancer, active in adults, and that the stabilities of the *toy^{hdl}* mRNAs are not significantly different from those of their wild-type counterparts. This result has been extended to embryos by in situ hybridization of a probe complementary to the 5′ portion of the *toy* mRNA that is not deleted by the *toy^{hdl}* deficiency. No difference in the patterns of *toy* transcripts was observed between homozygous *toy^{hdl}* and wild-type embryos (not shown). In contrast, when a probe covering the deleted exons 6-8 was used, a quarter of the embryos derived from heterozygous parents displayed no signal. It follows that none of the *toy* enhancers active in embryos or adults is affected by the *toy^{hdl}* deficiency. Moreover, we expect that the mutant produces a Toy^{hdl} protein that includes the N-terminal paired-domain but is truncated in the homeodomain (Fig. 3C).

Toy activates *ey* transcription in the embryonic anlagen of the eye-antennal discs

It has been proposed that the Toy protein activates *ey* in the eye developmental pathway. In the absence of *toy* mutants, this proposal was based (i) on the observation that ectopic eye formation induced by ectopic expression of *toy* in leg discs was dependent on a functional *ey* gene, and (ii) on in vitro binding studies of Toy to sites in a minimal *ey* enhancer whose activation of a reporter gene in the eye-antennal primordia was reduced upon mutation of the Toy binding sites (Czerny et al., 1999). Although these results indicate that the Toy protein activates the *ey* gene in the eye primordia, they do not prove it for two reasons. First, studies on ectopic eye formation do not strictly provide information about the normal pathway of eye formation, particularly since ectopic eye formation is possible in the absence of *toy* expression (Czerny et al., 1999). Second, mutation of the Toy binding sites in the minimal *ey* enhancer did not eliminate reporter gene expression in the entire eye-antennal primordium, but strong expression was still observed in its posterior portion, presumably where the eye primordium is located, perhaps because the minimal enhancer did not include all *cis*-regulatory elements required for proper expression of the *ey* gene during eye development (Czerny et al., 1999). Moreover, as evident from the *toy^{hdl}* phenotype (Fig. 1A,B), Toy is required not only for eye formation, but for the development of the entire eye-antennal disc and exhibits a phenocritical period at the time when the eye-antennal anlage forms and begins to express *ey* (Fig. 2).

The identification of deficiencies uncovering the *toy* gene and of *hdl* as a *toy* mutation (Fig. 3A,B), however, allows us to perform a more critical test of whether *toy* acts upstream of

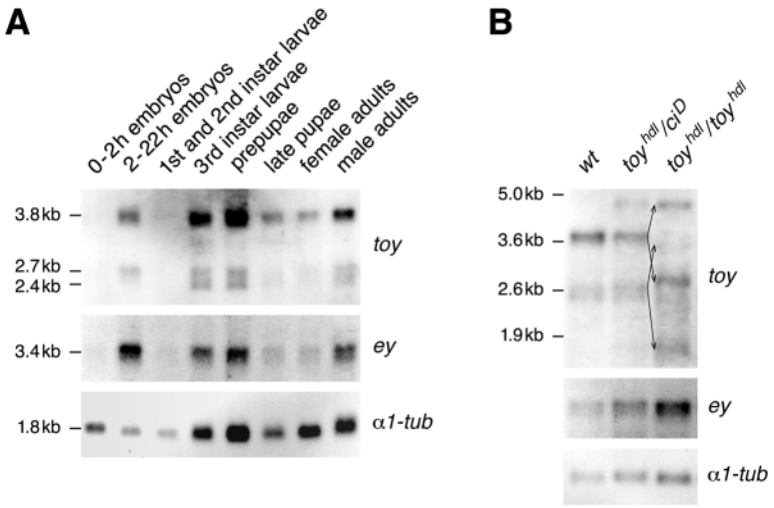

Fig. 4. Northern blot analysis of wild-type *toy* and *ey* mRNAs and of *toy^{hdl}* mRNAs. (A) Developmental profiles of wild-type *toy* and *ey* mRNAs. A northern blot of poly(A)$^+$ RNA, isolated from wild-type embryos, larvae and pupae of the stages indicated, and from female and male adults, was analyzed by autoradiography after successive hybridizations with ^{32}P-labeled 1.73 kb *toy* cDNA (top), 2.2 kb *α1-tubulin* genomic DNA for reference (bottom; Theurkauf et al., 1986), and 2.85 kb *ey* cDNA (middle). Sizes of mRNAs were calibrated with the same markers shown in B. (B) Northern blot analysis of *toy^{hdl}* mRNAs. A northern blot of poly(A)$^+$ RNA, isolated from adult flies (raised at 19°C) of the genotype indicated, was analyzed as described in A. Putative poly(A) addition signals consistent with the wild-type (*wt*) and *toy^{hdl}* mRNA sizes observed include the canonical AATAAA at positions 535 and 1633, and the non-canonical CATAAA at position 180 of the last exon of *toy* (cf. Fig. 3B). Arrows indicate which mutant mRNAs presumably are derived from which wild-type mRNAs by the use of the same poly(A) addition sites. RNA size markers shown were produced by the RiboMark Labeling System (Promega).

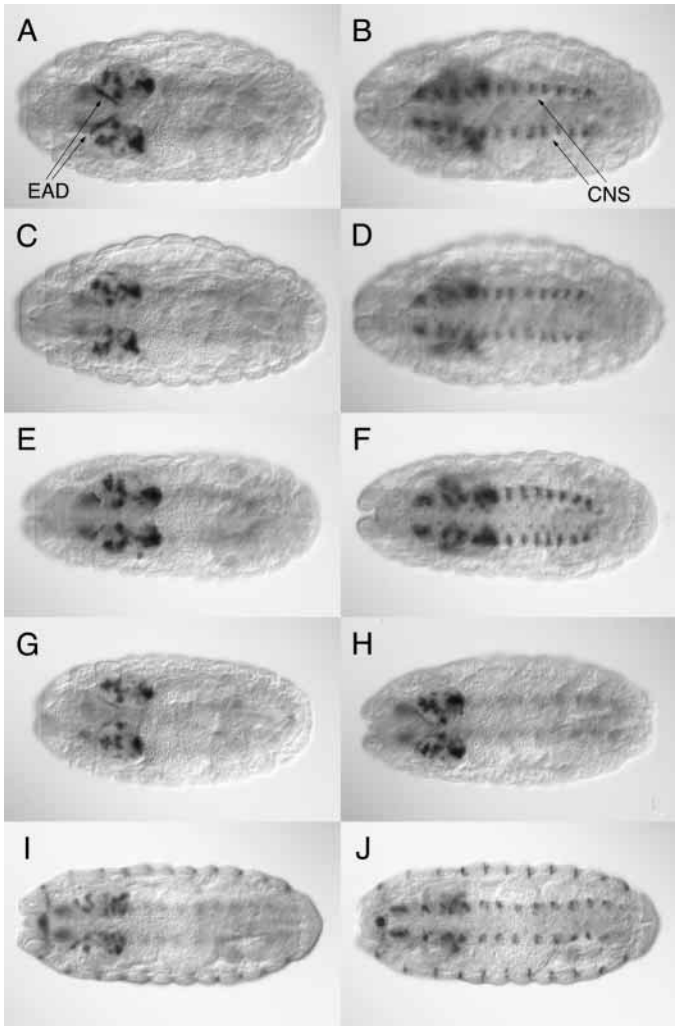

Fig. 5. Toy- and temperature-dependent transcription of *ey* in the eye-antennal anlagen. A-F are dorsal views of the same embryos focused in two different horizontal planes on *ey* transcripts in the eye-antennal primordia (left) or the CNS (right). (A-H) Transcription of *ey* in the eye-antennal primordia depends on Toy and temperature. Transcript levels of the *ey* gene, assayed by in situ hybridization with a DIG-labeled antisense RNA probe extending from exon 3 to 9 of the *ey* gene, in late stage 16 embryos derived from $toy^{hdl}/l(4)2C2$ (A-F), or $Df(4)spa^{66}/ci^{D}\ spa^{pol}$ parents (G,H) are normal in the eye-antennal primordia (EAD) of embryos with one or two wild-type copies of the *toy* gene (A,B), but clearly reduced in those of homozygous toy^{hdl} (C-F) and $Df(4)spa^{66}$ (G,H) embryos at 25°C (C,D,G) and 18°C (E,F,H). (I,J) Ectopic *ey* transcripts in homozygous or heterozygous ey^{D} embryos. Transcripts of the ey^{D} gene, detected by in situ hybridization of a probe specific for exons 1-5 of the *ey* gene, are shown in two different horizontal focal planes as ventral views of a late stage 16 embryo at 18°C derived from $ey^{D}/l(4)2C2$ parents. 18 out of 120 late stage 16 embryos developing at 25°C, and 9 out of 63 such embryos at 18°C showed strong ectopic expression of *ey* transcripts as in J.

Fig. 6. ey^{D} is a mutation in the *ey* gene. (A) The ey^{D} mutation is a translocation from the second chromosome into the fifth exon of the *ey* gene. At the top, the exons of the *ey* mRNAs (coding region in black, untranslated leader and trailer in white) are mapped with respect to the *Eco*RI sites of genomic *ey* DNA, which have been derived from genomic and cDNA sequences provided by FlyBase (Hauck et al., 1999) and from 5′-RACE products obtained from poly(A)$^{+}$ RNA of 0- to 20-hour-old embryos raised at 25°C. Below, the corresponding *Eco*RI map of the ey^{D} chromosome is illustrated. The ey^{D} mutation is shown to consist of an insertion after the 305th bp of exon 5, replacing the adjacent 320 bp. The insertion, which is a large reversed repeat of the 23D1,2 to 24BC region from the second chromosome, has been characterized by mapping and sequencing genomic clones isolated from an ey^{D} library in λ DASH II. The *Eco*RI map of two of these clones, EYD.1 and EYD.36, covering the proximal and distal breakpoint of the insertion, respectively, are shown at the bottom. Note that the ends of the large reversed repeat (hatched) are not identical but that the distal end extends 779 bp further into 24BC (stippled), while the proximal end includes a *roo* transposon, inserted at the indicated location. A 327 bp insertion (bearing no similarity to sequences of known genomes) in the ey^{D} chromosome, close to the distal end of the large insertion (190 bp downstream of the 5′ end of 'intron 5'), is indicated by a small triangle. R, *Eco*RI. (B) The ey^{D} insertion generates a truncated Ey protein. The wild-type Ey protein of 898 amino acids, including a paired-domain P and *prd*-type homeodomain H (Quiring et al., 1994), is compared to the truncated Ey^{D} protein resulting from the 2nd chromosome insertion into exon 5 of the *ey* gene. The truncated protein consists of 346 amino acids and includes the N-terminal paired-domain and a Ser/Thr-rich domain (28/66 amino acids), possibly an activation domain, but no homeodomain. Its 32 C-terminal amino acids are encoded by the inserted sequences of the second chromosome (black). The positions of introns are indicated by arrowheads. (C-E) Characterization of the ey^{D} mutation, a translocation of the second chromosome into the *ey* gene. In situ hybridization to polytene chromosomes of $ey^{D}/ci^{D}\ spa^{pol}$ late third instar larvae with the DIG-labeled probes indicated in A that are specific for the ends of the 2nd chromosome insertion (C), the 5′ end (D), and the 3′ end (E) of the *ey* gene. The inserts in C show enlarged views of the regions of hybridization on the second chromosome (lower left) and at the ends of the insertion on the fourth chromosome (upper right).

ey during normal eye and head development: the analysis of the expression of the *ey* gene in the eye-antennal primordia of *toy* mutant embryos at the end of the phenocritical period (Fig. 5C-H). Transcription of *ey* was strongly reduced, though not completely eliminated, in the eye-antennal primordia of most toy^{hdl} as compared to wild-type embryos when they developed at 25°C (compare Fig. 5C with 5A). If development occurred at 18°C, however, *ey* transcript levels were not reduced as much, but still clearly reduced in a third of the toy^{hdl} embryos (Fig. 5E) and indistinguishable from wild type in the remaining toy^{hdl} embryos. These findings prove that *toy* acts upstream of *ey* in the eye-antennal anlage at the time of the initial *ey* activation. In addition, they provide a simple explanation for the temperature sensitivity of the toy^{hdl} allele since the fractions of strong (Fig. 1A,B) and less severe headless phenotypes (Fig. 1C-E) correspond well to the fractions of embryos exhibiting reduced levels of *ey* transcripts in the eye-antennal primordia at the high (Fig. 5C) and low temperature (Fig. 5E). We conclude that *ey* transcription in the eye-antennal primordia of toy^{hdl} embryos is temperature-dependent and that the most severe headless phenotypes arise only if transcriptional activation of the *ey* gene in the eye-antennal primordia does not exceed a threshold level that is clearly detectable (Fig. 5C).

In contrast to *ey* transcription in the eye-antennal primordia,

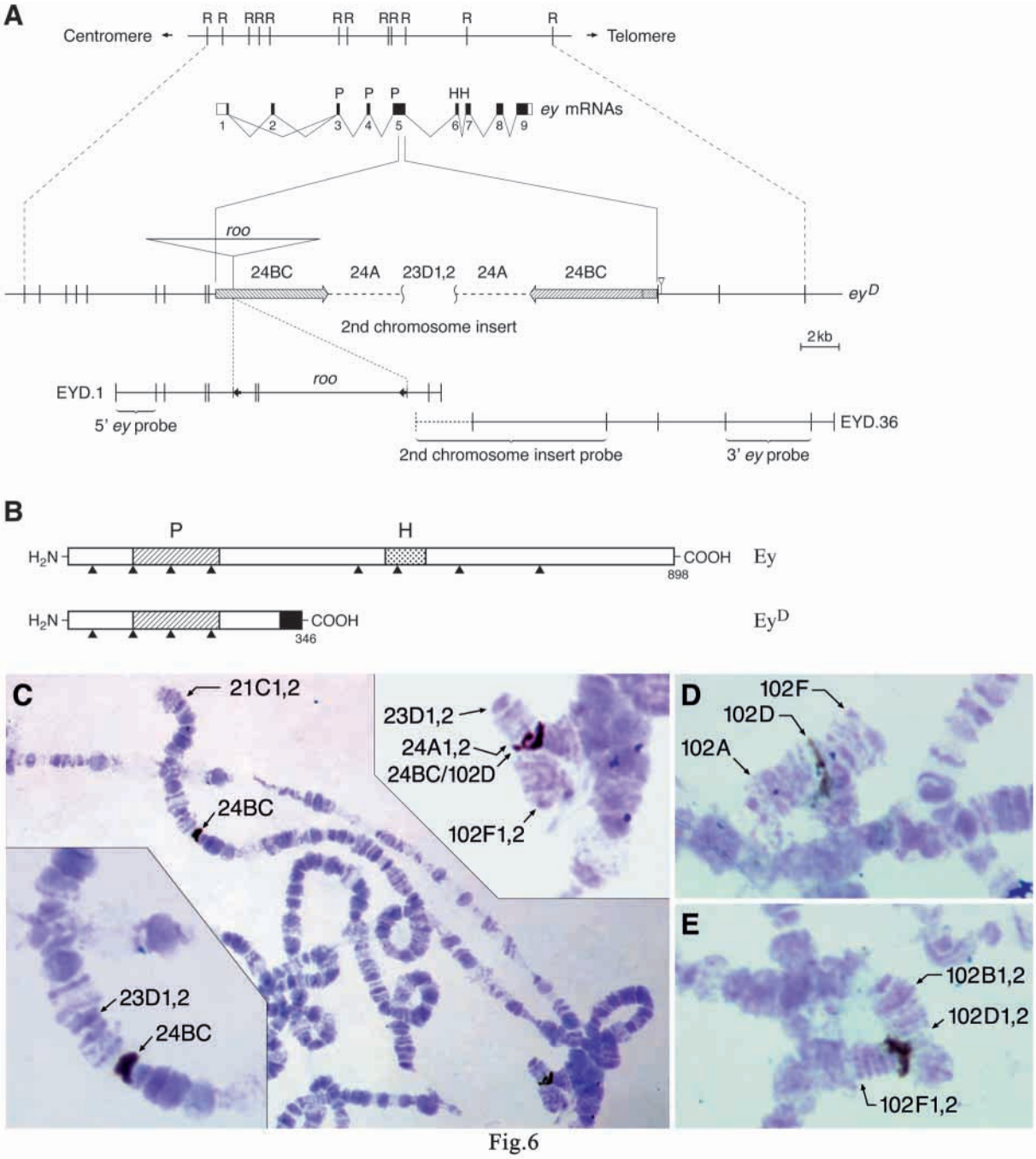

Fig.6

transcription of *ey* in the CNS appears to remain unaffected in *toy*^{hdl} embryos (Fig. 5B,D). This confirms an earlier conclusion based on the observation that *ey* transcripts precede those of *toy* and display a pattern different from *toy* transcripts in the ventral nerve cord (Czerny et al., 1999).

Temperature-dependent activation of *ey* transcription in eye-antennal anlagen in the absence of Toy protein

The temperature dependence of *ey* transcription in the eye-antennal primordia of *toy* mutants does not prove that the Toy^{hdl} protein is the cause of the temperature sensitivity of the headless phenotype. It is possible that even in the absence of Toy protein activation of *ey* transcription remains temperature dependent. To test this possibility, we examined *ey* expression in embryos homozygous for *Df(4)spa*^{30} or *Df(4)spa*^{66}, both of which fail to express *toy* transcripts (Materials and Methods). Surprisingly, in all homozygous mutant embryos the level of *ey* transcripts was similarly reduced as in *toy*^{hdl} embryos, but not eliminated, and still depended on temperature to the same degree (Fig. 5G,H). It follows that it is not the truncated Toy^{hdl} protein that is temperature dependent, but rather the activation of *ey* transcription in the absence of a functional Toy protein. This conclusion is consistent with our finding that similar fractions of homozygous *toy*^{hdl} pharates and of *toy*^{hdl} pharates transheterozygous for one of the two deficiencies show a

headless phenotype when tested at the same temperature between 18°C and 28°C. We conclude that toy^{hdl} behaves as a null allele with respect to the activation of *ey* at the phenocritical stage as well as with regard to the severity of the resulting headless phenotypes.

ey^D is an insertion interrupting the *ey* transcript

Since the severity of the headless phenotype correlates inversely with the level of *ey* transcripts in the eye-antennal primordia, we would expect that strong *ey* mutants also produce headless pharates if *toy* acts mainly through *ey* in eye-antennal development. None of the two defined *ey* mutants, ey^2 and ey^R, exhibits, however, a headless phenotype (Bridges, 1935a; Quiring et al., 1994; Halder et al., 1998), possibly because both are viable hypomorphs, whereas *ey* null alleles, all of which have been lost, are pupal lethals (Hochman, 1976). An additional putative *ey* mutation, ey^D, X-ray-induced by Muller in 1927 and mapped to the fourth chromosome (Patterson and Muller, 1930), has not been analyzed at the molecular level. Although its allelism to *ey* has been questioned (Bridges, 1935a), it is pupal lethal (Patterson and Muller, 1930; Bridges, 1935b) and has been characterized to be associated with the translocation of about a dozen chromosomal bands that are inserted as reversed repeat into the region 102D of the fourth chromosome (Bridges, 1935b) (Fig. 6C-E), known to include the *ey* locus (Quiring et al., 1994). Moreover, complementation analysis indicated that ey^D might be allelic to *ey* (Patterson and Muller, 1930) (Fig. 3A).

To test if ey^D is a lethal mutation of the *ey* gene, we isolated *ey* DNA clones from a genomic library of ey^D mutants and compared their *Eco*RI restriction maps to that of the wild-type *ey* locus (Quiring et al., 1994; Hauck et al., 1999). This analysis revealed that the large insertion on the fourth chromosome of ey^D mutants indeed interrupts the *ey* gene (Fig. 6A). Sequence analysis of mutant clones covering the breakpoints of the insertion showed that the *ey* gene is interrupted in exon 5 and that the adjacent 320 bp of exon 5 are replaced by a large insertion originating from the second chromosome (Fig. 6A,C). This insertion consists of a large reversed repeat (Bridges, 1935b), whose point of inversion is at 23D1,2 (Fig. 6C). Further analysis (Fig. 6D,E) showed that the *ey* gene is transcribed towards the telomere (Fig. 6A).

ey^D is transcribed and generates a truncated Ey^D protein that includes the paired-domain but lacks the homeodomain

In situ hybridization demonstrates that in about half of the homozygous ey^D embryos *ey* transcripts of the first five exons are as abundant in the eye-antennal primordia as in wild-type embryos, while in the other half they are reduced to about half the wild-type level, both at 18°C and 25°C (not shown). In contrast, no transcripts of the exons downstream of the ey^D insertion are detectable in homozygous ey^D embryos (not shown). These findings imply that the insertion has no strong effect on the embryonic activities of the *ey* enhancers and that the stability of the truncated ey^D transcripts is comparable to that of wild-type *ey* mRNA, independent of temperature within the examined range. It is therefore reasonable to assume that ey^D transcripts are translated to produce a truncated Ey protein that includes the N-terminal paired-domain and a Ser/Thr-rich domain, possibly an activation domain, but lacks all C-terminal domains, including the homeodomain (Fig. 6B).

In about a fifth of the homozygous or heterozygous ey^D embryos, the first five exons of *ey* were ectopically expressed in a metameric pattern of 11 lateral, later ventrolateral, posterior segmental stripes after germband retraction (Fig. 5I,J). Hence, a nearby enhancer of the inserted second chromosome sequences seems to activate the ey^D gene at low penetrance.

Homozygous ey^D pharate adults are headless

The homozygous ey^D condition is lethal (Patterson and Muller, 1930) with a lethal phase during the pupal stage (Bridges, 1935b), in agreement with the lethal phase of other strong *ey* alleles (Hochman, 1976) that, however, were lost. To investigate if homozygous ey^D mutants display a headless phenotype as expected, we dissected ey^D pharate adults from their pupal case and examined them by scanning electron microscopy. We found that indeed about half of these pharates showed a strong headless phenotype (Fig. 7B,C) missing all or most structures derived from wild-type eye-antennal discs (Fig. 7A), in agreement with an earlier report (Arking et al., 1975). The remaining pharates developed a considerable portion of the head and antennae, yet no eyes, and exhibited phenotypes most of which were stronger than the eyeless phenotype shown in Fig. 7D.

Both ey^2 and ey^R, shown to consist of transposon insertions into the eye-specific enhancer of *ey* (Quiring et al., 1994), on average enhance the heterozygous ey^D phenotype to produce smaller eyes (cf. Fig. 7E,F with 7G), but never headless flies. This finding strongly suggests that these two *ey* alleles are not null alleles, but retain some functions in developing eye-antennal discs even though they seem not to be expressed in these discs (Quiring et al., 1994) (our unpublished observation). The dominant phenotype of ey^D/+ adults whose eye size is moderately reduced (Fig. 7G) is probably the result of a dominant-negative effect of the truncated Ey^D protein that inhibits the wild-type Ey protein by competing for its targets during larval eye development, while haplo-insufficiency of *ey* is excluded by the wild-type phenotype of *Df(4)BA*/+ adults that are hemizygous for *ey* (Fig. 3A). Competition of Ey^D with targets of Toy appears improbable because the similarly truncated Toy^{hdl} protein does not exhibit a dominant-negative effect.

In combination with nearby deficiencies, some of which delete the *toy* gene but none the *ey* gene (Fig. 3A), the phenotype of heterozygous ey^D flies is affected in various ways. Thus, the size of the eye in $ey^D/Df(4)spa^{30}$ flies varies over a wider range than, but on average is similar to, that of ey^D/+ flies (Fig. 7H), while $Df(4)spa^{66}$ reduces the average eye size of heterozygous ey^D flies to about 10% of wild type (not shown). In contrast, $Df(4)spa^{47}$, which does not delete *toy* (Fig. 3A), seems to suppress the ey^D/+ phenotype significantly (not shown). These effects on the eyeless phenotype may result in part or entirely from different genetic backgrounds. For example, continued selection for small eye sizes among *y w*; $ey^D/Dp(1;Y;4)y^+$, spa^{pol} offspring obtained from repeated crossings inter se, yields increasing numbers of flies that have no eyes (Fig. 7I). Such variable effects of the genetic background on the ey^D/+ phenotype may explain why ey^D could not be identified unambiguously as an allele of *ey* by mere genetics.

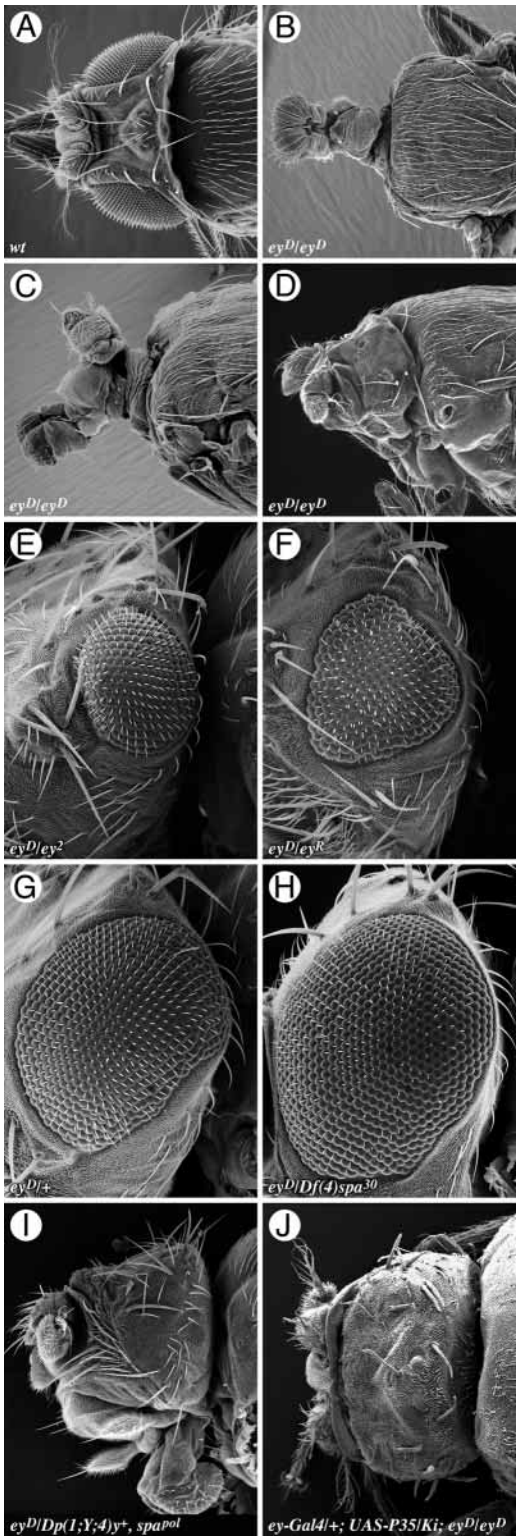

Fig. 7. Headless phenotype of ey^D pharates and their partial rescue by inhibition of apoptosis. Scanning electron micrographs of the anterior portion (A-D,I,J) or left eyes (E-H) of pharate (B-D) or viable (A,E-J) adults of the genotype indicated are compared. Note that, in contrast to the headless phenotype of toy^{hdl} flies, the penetrance and expressivity of the headless phenotype of ey^D pharates is the same at 18°C and 25°C with about 50% of the pharates exhibiting no (B) or only few (C) structures derived from the eye-antennal discs, while the phenotype of most pharates is much stronger than that shown in D. The variability of heterozygous ey^D phenotypes (E-I) presumably reflects a strong influence of the genetic background as illustrated by the eyeless phenotype obtained after several generations of selections for small eyes (I). Expression of the baculovirus P35 protein, an inhibitor of apoptosis (Hay et al., 1994), in eye-antennal discs is able to rescue more than half of the homozygous ey^D flies to viable adults. These flies possess both antennae, no eyes, but usually all three ocelli (J). (A,B,J) Dorsal, (C,D-I) lateral views, anterior is to the left.

Rescue of ey^D headless flies to viable eyeless adults by inhibition of apoptosis

Since about half of the ey^D pharates lacked most derivatives of the eye-antennal discs, we investigated whether inhibition of apoptosis by expression of the baculovirus P35 protein (Hay et al., 1994), under the indirect control of the eye-antennal specific enhancer of the *ey* gene, had any effect on eye-antennal disc development. Astonishingly, more than half of such ey^D flies are rescued by *ey-Gal4>UAS-P35* to viable adults that are eyeless, but have developed most other head structures derived from the eye-antennal discs, usually including all three ocelli (Fig. 7J). The heads of all these flies are rescued since their phenotypes are much weaker than the weakest ey^D phenotype (Fig. 7D). These results imply that the headless phenotype of ey^D mutants is the result of considerable cell death in the eye-antennal disc, a process that is inhibited by the wild-type Ey protein.

DISCUSSION

We have shown that an old mutant, *l(4)8*, induced by X-ray in 1957 by Gloor and Green (Hochman et al., 1964), displays a headless phenotype. This toy^{hdl} allele turned out to be the first mutant allele of the *Pax* gene *toy*, previously isolated as a paralog of the *ey* gene and proposed to activate *ey* to initiate eye development (Czerny et al., 1999). The dramatic phenotype of toy^{hdl} mutants, however, implies that *toy* is not only necessary for proper development of the eye, but for that of all structures derived from the eye-antennal disc. Moreover, we have shown that the same is true for the *ey* gene by demonstrating that ey^D is an allele of *ey* and much stronger than any *ey* alleles previously characterized (Quiring et al., 1994). The headless phenotype of ey^D pharates results from the induction of apoptosis in the eye-antennal disc, as evident from its rescue by the expression of the baculovirus P35 protein in eye-antennal primordia and discs. Our findings are consistent with an old conclusion that “at no time in development (except at the very end) is there a group of identified and committed cells which will give rise to the eye and to nothing else” (Martinez-Arias and Lawrence, 1985).

Activation of *ey* in eye-antennal primordia of *toy* mutants is temperature-dependent

Temperature shift experiments with toy^{hdl} mutants show that the headless phenotype critically depends on the absence of Toy protein activity during stages 12-16 of embryogenesis (Fig. 2). In toy^{hdl} mutants, up to 80% of the pharate adults are headless at 28°C, whereas this phenotype is nearly completely

suppressed at 18°C. Shifting the temperature down to the permissive temperature at the end of stage 16 demonstrates that after this stage Toy^{hdl} is unable to provide any function that would be able to rescue the headless phenotype, while shifting the temperature up to the non-permissive temperature at this time shows that Toy^{hdl} can provide all the functions necessary, if any, to rescue the headless phenotype at all temperatures after stage 16. It follows that the temperature-sensitive function of Toy^{hdl} with regard to the headless phenotype is restricted to the phenocritical period during stages 12 to 16.

Homozygous *ey^{D}* pharates exhibit the same headless phenotype as *toy^{hdl}* mutants. Since we have demonstrated that *ey^{D}* is a strong allele of the *ey* gene, we conclude that the truncated Ey^{D} protein, if translated from the *ey^{D}* mRNA, is unable to provide the functions necessary for eye-antennal disc development. It was therefore important to know if *ey* transcription depended on Toy during the phenocritical period, as had been shown previously for ectopic eye formation, but could not be tested for normal eye development because of the lack of *toy* mutants (Czerny et al., 1999). We found that *ey* transcription indeed depends on *toy* activity. Surprisingly, however, even in the absence of Toy protein *ey* transcription, (i) remains temperature-dependent during the phenocritical period, and (ii) is not completely eliminated. Hence, the observed temperature-sensitivity of the headless phenotype of *toy^{hdl}* mutants is not a property of the truncated Toy^{hdl} protein, but rather of the transcriptional activation of the *ey* gene in the absence of a functional Toy protein.

Does Toy serve to stabilize a temperature-dependent activation complex on the eye-antennal enhancer of the *ey* gene?

We propose that during the phenocritical period, in addition to Toy, other transcription factors bind to the eye-antennal enhancer of the *ey* gene to activate its transcription in the eye-antennal primordia. In the absence of Toy, these factors are able to activate *ey* transcription sufficiently at low but not at high temperatures to ensure normal eye-antennal development. The simplest explanation for this temperature-sensitive activation of *ey* is that formation of the transcription factor complex bound to the eye-antennal enhancer or its activation of the basal transcription machinery becomes temperature-dependent in the absence of Toy protein. Thus, the main function of Toy is to stabilize this transcriptional activator complex on the eye-antennal enhancer of *ey*. It is interesting to note that three Toy binding sites have been found in the eye-antennal enhancer of *ey*, two of which are immediate neighbors (Czerny et al., 1999). Truncated Toy^{hdl} probably binds with similar affinity to these sites as wild-type Toy protein (Punzo et al., 2001). However, its lack of C-terminal activation domains may fail to stabilize its own binding and that of cofactors as a result of which the basal transcription machinery is not efficiently recruited and activated. The fact that a third of the *toy^{hdl}* mutants die during larval stages and that *toy^{hdl}* mutants that show normal eye-antennal development still die as pharate adults shows that Toy is more strictly required for the activation of other enhancers necessary for the development of viable adults.

Eye-antennal development depends on a delicate balance of *ey* activation in the eye-antennal primordia

Interestingly, *ey* transcription is not abolished, but reduced levels of *ey* transcripts remain detectable in eye-antennal primordia of *toy^{hdl}* embryos, even at temperatures at which most of them develop to headless pharates. This finding implies that there is a delicate balance of *ey* activation for inducing eye-antennal development. If transcript levels do not exceed a relatively high threshold value, the program for eye-antennal disc development cannot proceed. This delicate balance is particularly evident when it is temperature-dependent in the absence of a functional Toy protein, for example, in *toy^{hdl}* mutants. In many instances, unequal *ey* transcript levels have been observed in the left and right eye-antennal primordia of *toy^{hdl}* embryos. Accordingly, *ey* transcripts may surpass the threshold in only one of the two eye-antennal primordia and thus give rise to pharates with only one half of the head developing normally (Fig. 1C). Even within the eye-antennal primordium, *ey* transcript levels may vary in *toy^{hdl}* mutants (Fig. 5E) and thus explain phenotypes like the cleft-head (Fig. 1D).

Partial redundancy of Toy and Ey functions

In *toy^{hdl}* mutants, reduced yet detectable (moderate) levels of *ey* transcripts in eye-antennal primordia are unable to rescue the headless phenotype. This is evident from the reciprocal correlation between *ey* transcript levels in eye-antennal primordia during the phenocritical period and the fraction of headless pharates at different temperatures of development. In contrast, *ey^{2}* mutants, in which no *ey* transcripts have been detected in eye-antennal primordia, never display a headless phenotype and mostly have eyes of only slightly reduced size (Quiring et al., 1994) (our unpublished observation), whereas *ey^{D}* mutants show a high penetrance of headless pharates. The following considerations illustrate that this apparent contradiction is resolved by the assumptions that (i) Toy and Ey share partial functional redundancy in eye-antennal disc development, and (ii) *ey^{2}* expresses very low levels of wild-type Ey protein whose mRNA escapes detection (Fig. 8). In the absence of functional Ey protein, as in *ey^{D}* mutants, normal levels of functional Toy protein rescue the headless phenotype at low efficiency. In *ey^{2}* mutants, however, very low levels of functional Ey protein are sufficient to rescue the headless phenotype completely in the presence of normal Toy levels and are even able to promote nearly normal eye development (our unpublished results). In contrast, in the absence of functional Toy protein, as in *toy^{hdl}* mutants, much higher levels of *ey* transcripts are necessary to rescue the headless phenotype, which is achieved more efficiently at lower temperatures. Consequently, the headless phenotype is observed only in the complete absence of functional Ey protein or in the absence of functional Toy if Ey does not exceed a moderate level.

This partial redundancy of Toy and Ey functions implies that Toy does not act exclusively through the activation of *ey*, but also in a pathway parallel to *ey*, to promote eye-antennal disc development (Fig. 8). Such an *ey*-independent pathway downstream of *toy* may include the *optix* gene, a paralog of the *sine oculis* gene (Seimiya and Gehring, 2000) which is in turn a target of *ey* (Halder et al., 1998; Niimi et al., 1999), and the *eyg* gene (Jun et al., 1998; Gehring and Ikeo, 1999).

Rescue of *ey^{D}* mutants to viable adults by the inhibition of apoptosis in eye-antennal discs

The most crucial function of *ey* and most sensitive to the level

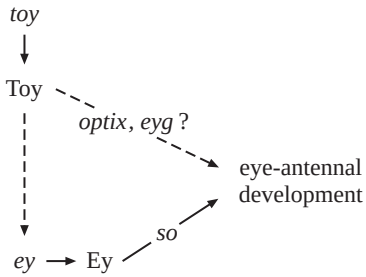

Fig. 8. Redundant functions of Ey and Toy in eye-antennal development. Normal head (eye-antennal) development can occur in the absence of Toy if Ey levels are sufficiently high, such as is mostly the case in toy^{hdl} mutants at 18°C, or in the presence of very low Ey levels if Toy levels are normal as in ey^{2} mutants. Conversely, headless phenotypes are observed in the absence of Toy and at moderate Ey levels, as in most toy^{hdl} mutants at 28°C, or in the absence of functional Ey as in ey^{D} mutants. Broken lines indicate where requirement for eye-antennal development is not absolute because it can be compensated by sufficiently high levels of Ey. It is unclear if the pathway of Toy that is parallel to the *ey* pathway leads through *optix* and/or *eyg*, as the diagram suggests. Moreover, additional Toy and Ey functions that are not mediated through *so* and *optix/eyg*, respectively, exist to support proper eye-antennal development. Such Toy and Ey functions, like the inhibition of apoptosis, as shown here, or the activation of the cell cycle (Jiao et al., 2001), are required in eye-antennal primordia of stage 12-16 embryos, long before these genes are activated in eye-antennal discs (Kumar and Moses, 2001).

of Ey during eye-antennal development is the inhibition of cell death. This became evident from a successful attempt to rescue the ey^{D} headless phenotype by the expression in eye-antennal discs of the baculovirus P35 protein, an inhibitor of apoptosis (Fig. 7J). Astonishingly, P35 is able to rescue more than half of the ey^{D} embryos to viable adults with normal head structures, with the exception of the eyes that still do not develop.

The fact that inhibition of apoptosis in ey^{D} eye-antennal discs is unable to rescue eye development argues that additional functions of *ey* during eye-antennal development are required and restricted to the eye disc, in agreement with its expression pattern in eye-antennal discs (Quiring et al., 1994) and a recent analysis (Kumar and Moses, 2001). It has been shown that interference with the program of eye-antennal development by ectopic expression of transcription factors also generates headless pharates if interference is restricted to exactly the same phenocritical period observed here (Jiao et al., 2001). However, in this case interference could not be antagonized by P35 expression, but only by overexpression of Ey, CycE or Myc. It was concluded that one of the earliest functions of *ey* must be the activation of the cell cycle (Jiao et al., 2001). Combining this conclusion with the results reported here, we propose that in the presence of developmental pathway interference it is impossible to obtain rescue by the inhibition of apoptosis in the absence of cell cycle activation, whereas inhibition of apoptosis appears to suffice in ey^{D} mutants, possibly because the truncated Ey^{D} and/or the Toy protein are able to activate the cell cycle.

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