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shaven and *sparkling* are mutations in separate enhancers of the *Drosophila Pax2* homolog

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SUMMARY

We have previously shown that the *sparkling* gene, which like mammalian *Pax2* plays an important role in eye development, is encoded by the *Drosophila* homolog of *Pax2*. Here we demonstrate that *D-Pax2* also encodes the *shaven* function, which is crucial during bristle development. Both *sv* and *spa* alleles, previously thought to represent different genes, are mutations in two widely separated enhancers of *D-Pax2*. The *sv* function of *D-Pax2* acts in at least two distinct steps of mechanosensory bristle development: the specification of the alternative fate of shaft as opposed to socket cell, and later the differentiation of the shaft cell.

Key words*: shaven*, *sparkling*, Enhancer mutations, *Pax2* homolog, Bristle development, *Drosophila*

INTRODUCTION

Recently, we have isolated the *sparkling* (*spa*) gene of *Drosophila* and shown it to encode a paired-domain transcription factor most closely related to the vertebrate Pax2/Pax5/Pax8 proteins (Fu and Noll, 1997). Similar to the vertebrate *Pax2* gene (Torres et al., 1996), *spa* plays an important role in eye development where it is required for the proper specification of cone and primary pigment cells in late larval and pupal eye discs (Fu and Noll, 1997). This is evident from spa^{pol} mutants in which lack of *spa* expression in cone and primary pigment cells leads to a severe disruption of the hexagonal lattice of ommatidia and hence to a rough eye phenotype. It was further shown that the spa^{pol} mutant phenotype results from the deletion of an enhancer located in the fourth intron and necessary to activate *spa* transcription in cone and primary pigment cells. We now demonstrate that the gene encoding the *spa* function in eye development also plays a decisive role in bristle development.

During evolution, new functions may evolve by gene duplication and the concomitant acquisition of a new enhancer (Li and Noll, 1994; Carroll, 1995; Xue and Noll, 1996). A functional diversification may also result if the protein coding region acquires an additional enhancer. Since each enhancer of such a gene specifies a distinct function, its alleles that have mutations in different enhancers are expected to complement each other and hence to behave genetically as separate genes. Classical examples are the genes of the bithorax complex (Lewis, 1978; Bender et al., 1983; Sánchez-Herrero et al., 1985; Peifer et al., 1987). Here we show that the closely linked mutant alleles *spa* and *shaven* (*sv*), thought to represent two separate genes (Hochman, 1971), result in fact from mutations in distinct enhancers controlling the expression of the same protein. If the mutations occur in the enhancer of *sv*, expression is reduced in sensory organ precursor cells (SOPs) and eliminated in shaft and glial cells derived from SOPs by asymmetric divisions. Accordingly, *sv* mutants show a reduction or absence of shafts, which is frequently accompanied by the conversion of a shaft into a socket cell and hence suggests that the specification of shaft as opposed to socket cell fate crucially depends on the *sv* function.

MATERIALS AND METHODS

General procedures

Standard procedures, such as isolation and Southern blot analysis of genomic DNA, screening of genomic libraries and in situ hybridization to salivary gland chromosomes, were carried out essentially as described by Maniatis et al. (1982) and Frei et al. (1985).

Characterization of *sv* and *spa* mutations

Genomic libraries were constructed from DNA of $Df(4)spa^{20}/ci^{D}$ spa^{pol} and $T(3;4)Ubx^{A}$, *cu kar* Ubx^{A} $spa^{A}/MKRS$ stocks in the λ DASH II vector (Stratagene) to isolate phages harboring the $Df(4)spa^{20}$ and the $T(3;4)Ubx^{A}$ spa^{A} breakpoints, respectively. Mutant DNA fragments of sv^{n}, sv^{35a}, and sv^{de} were isolated from genomic DNA of sv^{n}/sv^{n}, sv^{35a}/sv^{35a}, sv^{de}/ey^{D} stocks by PCR, by means of primers in the first intron (0.34 kb downstream of the first exon) of *D-Pax2* (5′-TGTTACGAGGTCATTGGAGCTT-3′) and 2.3 kb upstream of its transcriptional start site (5′-GTGCTATCGACTTGTTAAAGCC-3′). That the enlarged PCR fragment obtained from sv^{de}/ey^{D} DNA originated from the sv^{de} rather than from the ey^{D} chromosome was verified by PCR of $sv^{de}/Df(4)spa^{20}$ and $sv^{de}/Df(4)spa^{30}$ DNA. The

breakpoints of *Df(4)spa*^{20} and *T(3;4)Ubx*^{A} *spa*^{A} and sites of insertion in *sv*^{n}, *sv*^{35a}, and *sv*^{de} were precisely mapped with respect to the wild-type *D-Pax2* sequence by DNA sequencing with a DNA sequencer model 373A using dye terminators (Applied Biosystems Inc.).

DNA of *sv*^{n} and *sv*^{35a} stocks had identical insertions of the *blastopia* retrotransposon (Frommer et al., 1994) at identical sites and hence were indistinguishable as a result of a mix-up and loss of one of them, probably *sv*^{35a}, that must have occurred a long time ago. Sequencing of the *sv*^{de} insertion revealed that it is a defective *Tirant* element, a gypsy-like retrotransposon (Thomas et al., 1995; Moltó et al., 1996; Viggiano et al., 1997). All published and unpublished *Tirant* sequences are derived from defective retrotransposons. The defective *Tirant* element at *sv*^{de} is deleted from position 4316 to 7456 and harbors only the last three of six 102 bp repeats (639 to 946 is absent). This follows from a comparison with the sequence of a nearly complete *Tirant* element of 7818 bp which is truncated by only 709 bp at its 3′ end (sequence available as P1 subclone DS01096.1 from the Berkeley Drosophila Genome Project; BDGP). Accordingly, a lower limit of 8.4 kb for the size of an intact *Tirant* element may be derived from the four incomplete *Tirant* sequences. Sequencing of the *spa*^{1} insertion, mapped to the fourth intron (Fu and Noll, 1997), demonstrated its identity with the 7520 bp *yoyo* retrotransposon (sequence available as P1 clone DS01219, position 9180 to 1661, from the BDGP) related to the earlier described gypsy-like retrotransposon *yoyo* of the medfly (GenBank accession number U60529: Q. Zhou and D. Haymer, personal communication). The sequence of the polymorphic insertion in the last intron of *D-Pax2* revealed the presence of a 4.73 kb *Doc* retroposon (O'Hare et al., 1991); it had no detectable effect on the phenotype (Fu and Noll, 1997).

The reciprocal translocation between *Ubx* and *spa*, *T(3;4) Ubx*^{A} *spa*^{A}, is part of a complicated rearrangement that includes several additional inversions on the third, but none on the fourth, chromosome (Lindsley and Zimm, 1992). It allowed us to map the orientation of *spa* on the fourth chromosome by in situ hybridization to salivary gland chromosomes by means of genomic *spa* probes on either side of the breakpoints, because the distal probe is expected to generate two signals, one each on the third and fourth chromosome, whereas the proximal probe would generate only a single signal on the fourth chromosome. The reciprocal translocation has breakpoints in the last intron of *spa* (28 bp downstream from the 12-bp duplicated target site of the polymorphic insertion of a 4.73 kb *Doc* retroposon; cf. Fig. 3A) and in the large last intron of *Ubx* (3.8 kb upstream of the last exon). As a result, the right telomeric region of the fourth chromosome that includes the large 5′ portion of *spa* is fused to the large 5′ portion of *Ubx* and the short 3′ end of *spa* to that of *Ubx*. Sequencing locates the breakpoint of the *spa*^{A}(5′)/*Ubx*^{A}(5′) fragment within the sequence 5′-ACTAGTCTGAGGACA | GCGTCTCAGCTCCGG-3′ at coordinate 314581 of the *BX-C* sequence and the breakpoint of the reciprocal *spa*^{A}(3′)/*Ubx*^{A}(3′) fragment within the sequence 5′-ACTGCAGAATAATGC | AGCGCA | TAATTGTTTTATAGA-3′ at coordinate 314585 of the *BX-C* sequence (GenBank accession number U31961). While no deletion of any *spa* sequences resulted during the translocation, 3 bp of *Ubx* have been deleted and replaced by the 6 bp indicated at the *spa*^{A}(3′)/*Ubx*^{A}(3′) junction.

P-element-mediated rescue of *sv* and *spa* mutant phenotypes

Complete rescue of *spa*^{pol} mutants (Fig. 4E) was obtained by germline transformation with the P-element vector construct *Spe*I/*spa*-PCG2 harboring a *spa* transgene regulated by a 926-bp *Spe*I enhancer fragment of intron 4 (Fu and Noll, 1997).

For rescue of *sv*^{n} and *spa*^{pol} mutants, a *Hs-spa* construct was prepared by cloning a 2.8 kb *Eco*RI-*Xba*I fragment of cpx1 *spa*-cDNA, which includes the entire open reading frame of the Spa protein (Fu and Noll, 1997), downstream of the *hsp70* promoter and upstream of the *tubulin* trailer into the neighboring unique *Kpn*I and *Eco*RI sites of the pKB256 vector. This vector, which has been constructed and kindly provided by K. Basler, is a derivative of the P-element vector pW8 (Klemenz et al., 1987) which contains the *hsp70* heat shock promoter (an *Xba*I-*Kpn*I fragment consisting of the first 95 bp of the *hsp70* leader and 250 bp upstream sequences) and an adjacent 0.8 kb genomic tubulin fragment serving as transcriptional terminator.

For rescue of *sv* mutants, two additional constructs, *2.0-spa* and *6.7-spa*, were generated. The *2.0-spa* construct was prepared by replacing the *Xba*I-*Kpn*I fragment harboring the *hsp70* promoter in the *Hs-spa* construct with a 2.1 kb upstream and leader fragment consisting of 2.0 kb upstream region and adjacent 60 bp leader of *D-Pax2*. It was produced from the cognate genomic clone by PCR and the use of an upstream primer with an attached *Xba*I site (5′-CTAGTCTAGAGCTACCAGGCGAATTCTACAAA-3′) and of a leader primer with an attached *Kpn*I site (5′-GGGGTACCTGTTGGCTAAAAATCCACGAAGCA-3′). The *6.7-spa* construct was generated by replacing the 926-bp *Spe*I enhancer fragment and the 330 bp *spa* promoter of the *Spe*I/*spa*-PCG2 construct by a *Xba*I-*Bsi*WI fragment that extends 6.7 kb upstream from the transcriptional start site located a few bp upstream of the *Bsi*WI site. As a control, the 6.7 kb *Xba*I-*Bsi*WI upstream fragment was cloned into the polylinker of the pW6 vector (Klemenz et al., 1987) to prepare a construct that, in contrast to the *6.7-spa* construct, cannot rescue *sv*^{n} mutants.

For germline transformation of mutant stocks, the *Hs-spa* construct was injected into *w*^{1118}; *spa*^{pol} embryos whereas the *2.0-spa*, *6.7-spa* and the control constructs were injected into *w*^{1118}; *sv*^{n} embryos. Of all constructs several independent transgenic lines were obtained except for *Hs-spa* of which only one healthy line (*w*^{1118}; *P[w*$^{+}$; *Hs-spa]/CyO*; *spa*^{pol}) could be established, probably because the *Hs-spa* transgene is lethal unless it has integrated at a chromosomal site where its expression occurs at sufficiently low levels in the absence of heat shock.

Partial rescue of the *spa*^{pol} phenotype (Fig. 4F) was achieved by transferring *Hs-spa/+*; *spa*^{pol} early third instar larvae to 29°C for 2 days and returning them to 25°C until eclosure. Only slight rescue of the *spa*^{pol} phenotype was observed without temperature shift. *Hs-spa/+*; *sv*^{n} flies displayed complete rescue of the *sv*^{n} phenotype on the notum when grown at 25°C (Fig. 4K), indicating that the *Hs-spa* transgene of this healthy line is slightly leaky in the absence of heat shock and that relatively low expression of *Hs-spa* suffices to rescue the bristle phenotype in the notum of *sv*^{n} hypomorphs. In contrast, no rescue of eye bristles occurred in such flies, whereas partial rescue of eye bristles was observed when white pupae were shifted three times to 37°C for 20 minutes, interrupted by two 1-hour intervals at 25°C for recovery, and returned to 25°C until eclosure (Fig. 4L).

Scanning electron microscopy and immunohistochemistry

Scanning electron microscopy of adult flies was kindly carried out by Urs Jauch (Institut für Pflanzenbiologie, Zürich, Switzerland) or Thomas Gutjahr of our lab (Basler et al., 1991). Immunohistochemical staining for Spa protein in embryos and imaginal discs was as described by Fu and Noll (1997).

Mutagenesis and screen for *spa* alleles

In an F_1 mutant screen, males were mutagenized with EMS (Lewis and Bacher, 1968) and crossed to *ci*^{D} *spa*^{pol}/*ey*^{D} virgins. Their offspring were screened for rough eyes, and individuals with a rough eye phenotype were back-crossed to *ci*^{D} *spa*^{pol}/*ey*^{D} flies to establish balanced stocks of the *spa* mutant alleles over *ci*^{D} *spa*^{pol}. From about 60,000 F_1 offspring, four strong *spa* alleles, *spa*^{20}, *spa*^{30}, *spa*^{47} and *spa*^{66}, were generated, all embryonic lethal deficiencies.

Fly stocks

Mutant stocks of *Df(4)G/ci*^{D}, *ci*^{D} *spa*^{pol}/*ey*^{D}, *spa*^{pol}/*spa*^{pol}, *sv*^{n}/*sv*^{n}, *sv*^{35a}/*sv*^{35a}, *sv*^{de}/*ey*^{D}, *l(4)29/ci*^{D}, *l(4)39/ci*^{D} and *Df(4)38/ci*^{D} were

kindly provided by the Bloomington, Bowling Green and Umea stock centers and *T(3;4)Ubx^{A}*, *cu kar Ubx^{A} spa^{A}/MKRS* by Adelaide Carpenter.

RESULTS

Spa protein is expressed in developing larval and adult sensory organs

Expression and function of the Spa protein is not restricted to cone and primary pigment cells although these appear to be the only cells in *spa^{pol}* mutants in which *spa* transcription is abolished (Fu and Noll, 1997). During embryogenesis, Spa is expressed in the central nervous system (CNS) and in the peripheral nervous system (PNS) where it is observed in developing external sensory organs (Czerny et al., 1997) and chordotonal organs of the larva (Fig. 1E; Fu and Noll, 1997). Later, during late third instar larval and early pupal stages, Spa is not only detected in cone and primary pigment cells (Figs 1A and 2A) but also in all four precursor cells of the mechanosensory bristles of the developing pupal retina (Fig. 2A; Fu and Noll, 1997). At subsequent pupal stages, Spa expression is lost from socket cells and neurons whereas shaft and glial cells continue to express it (Fig. 2A). In addition, Spa protein is expressed in what appear to be SOPs of antennal, leg and wing discs (Fig. 1A-C). These cells include SOPs of chordotonal organs, such as those of Johnston's organ in the second antennal segment (Fig. 1A; Y. Shi and M. N., unpublished results), as well as of external sensory organs. Particularly, these last observations point to an additional role of the Spa protein in bristle development.

Intriguingly, there is a locus closely linked to *spa*, *sv* (Hochman, 1971), whose alleles are known to affect the development of bristles, causing shafts to be shortened or absent in adult flies (Lindsley and Zimm, 1992). Thus, our observation of Spa expression in developing bristle cells raises the hypothesis that *sv* and *spa* might represent distinct functions of one and the same transcription unit when expressed under the control of different enhancers. Below we will demonstrate that this is indeed the case.

Expression of Spa protein is abolished in shaft and glial cells of *sv^{n}* mid-pupal eye discs

Consistent with this supposition, we find that *spa* expression is lost in *sv* mutants. While Spa protein is not detectable in cone and primary pigment cells of *spa^{pol}* mutants, its expression in bristle cells appears unaffected (Fig. 2B). The situation is reversed in *sv^{n}* mutants: Spa protein is absent from nearly all shaft and glial cells, which both continue to express Spa in wild-type mid-pupal eye discs, but its expression in cone and primary pigment cells is the same as in wild type (cf. Fig. 2A and 2C). At earlier pupal stages, Spa protein is still expressed in all four bristle cells of *sv^{n}* eye discs (not shown), in agreement with the observation that *sv^{n}* flies display a hypomorphic *sv* phenotype (Lindsley and Zimm, 1992).

Two *sv* alleles carry insertions of retrotransposons in the upstream region of *spa*

That *sv* and *spa* are alleles of the same gene was also consistent with the result of a molecular analysis of mutant *sv* alleles. When the three available *sv* alleles, *sv^{n}*, *sv^{35a}* and *sv^{de}*, were examined for molecular lesions in, or close to, the *spa* transcription unit, all were found to carry insertions of transposable elements within a 0.25 kb *Eco*RI fragment about 1.6 kb upstream from the transcriptional start site of *spa* (Fig. 3A). While *sv^{n}* and *sv^{35a}* turned out to be identical insertions of the 5.0 kb *blastopia* retrotransposon (Frommer et al., 1994), a 5.1 kb *Tirant* element (Garrell and Modolell, 1990) is inserted in *sv^{de}*. Although this evidence strongly suggests that *sv* and *spa* are alleles of the same transcription unit, it is conceivable that *sv* and *spa* are adjacent or very closely linked genes and that Spa expression in bristle cells depends on the product of *sv*.

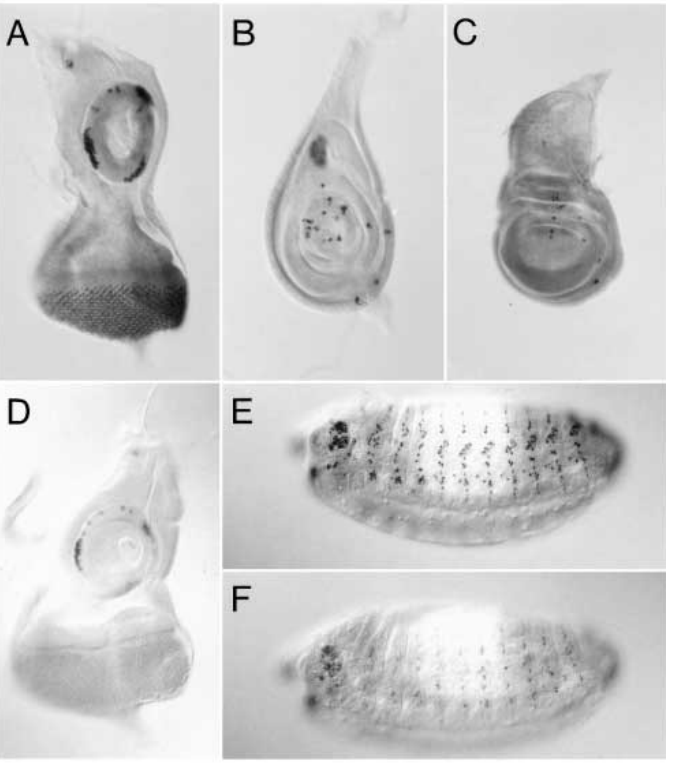

Fig. 1. Expression of Spa protein in precursors of larval and adult sensory organs and its reduction in *l(4)spa^{20}* mutants. Nuclear Spa protein was detected by immunohistochemical staining with a rabbit anti-Spa antiserum in third instar larval eye-antennal (A,D), leg (B), and wing discs (C), and in stage 14 embryos (E,F) of wild type (A-C), *l(4)spa^{20}/ci^{D} spa^{pol}* (D,E) and homozygous *l(4)spa^{20}* mutants (F). Only heterozygous offspring of the *l(4)spa^{20}/ci^{D} spa^{pol}* stock survive to the larval stage of the disc shown in D since both *ci^{D}* and *l(4)spa^{20}* are homozygous embryonic lethal. Note the expression of Spa in patches of cells whose positions correlate well with those of the precursors of Johnston's organ in the second antennal segment (A,D) and of the femoral chordotonal organs in the leg disc (B). In embryos (E,F), Spa expression is shown in developing external sensory organs and chordotonal organs, while its expression in the CNS is out of focus and hence not visible. Expression in both PNS and CNS is considerably reduced in homozygous *l(4)spa^{20}* (F) as compared to *l(4)spa^{20}/ci^{D} spa^{pol}* embryos (E), and both expression patterns can be easily distinguished from the disturbed pattern of homozygous *ci^{D} spa^{pol}* embryos obtained from the same *l(4)spa^{20}/ci^{D} spa^{pol}* parents. Imaginal discs of late third instar larvae are oriented with anterior end (A,D) or dorsal side (B,C) up. Ventrolateral views of embryos are shown with their anterior to the left and dorsal side up.

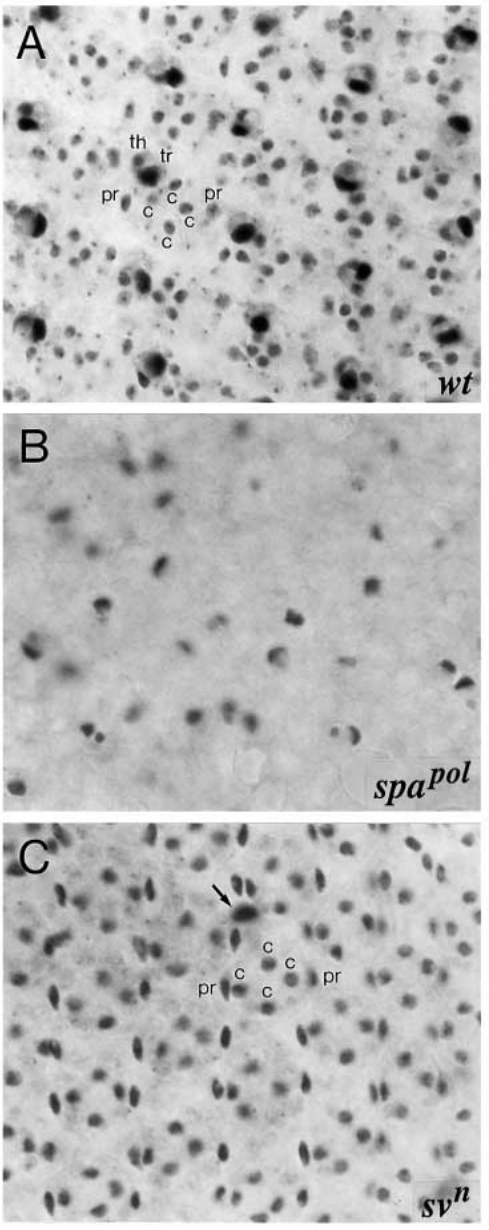

Fig. 2. Absence of Spa protein from bristle cells in mid-pupal eye imaginal discs of sv^n but not of spa^{pol} mutants. Spa protein was detected by immunohistochemical staining with a rabbit anti-Spa antiserum in nuclei of wild-type (A), homozygous spa^{pol} (B), and homozygous sv^n (C) eye discs 45 hours after puparium formation (APF) at 25°C. In wild-type eye discs (A), Spa protein is observed in cone (c) and primary pigment cells (pr) and in two of the four cells that will constitute a bristle, namely in precursors of trichogens (tr) or shaft cells and of thecogens (th) or glial cells. In mid-pupal eye discs of spa^{pol} mutants (B), Spa is expressed only in trichogens and thecogens but not in cone and primary pigment cells while the situation is reversed in eye discs of sv^n mutants (C), with few exceptions of shaft cells that still express Spa (arrow in C). Notice that by this stage shaft cells express considerably higher levels of Spa protein than glial cells and that, in contrast to an earlier pupal stage (24 hours APF), the spacing of bristle cells has become irregular in spa^{pol} mutants (cf. Fu and Noll, 1997). Different types of bristle cells have been identified by position and by double-labeling with the neural marker mAb22C10 (Zipursky et al., 1984).

Search for *spa* alleles that fail to complement *sv* alleles

To discriminate between these alternative possibilities, additional *spa* alleles were generated by mutagenesis with ethyl methane sulfonate (EMS) and tested for complementation with *sv*. Four *spa* alleles, $l(4)spa^{20}$, $l(4)spa^{30}$, $l(4)spa^{47}$ and $l(4)spa^{66}$, were obtained, of which none complemented sv^n. Three of these new *spa* alleles, $l(4)spa^{30}$, $l(4)spa^{47}$ and $l(4)spa^{66}$, are large deficiencies and thus unable to resolve the issue as they also fail to complement two lethals, *l(4)39* and *l(4)29*, mapped immediately proximal to *sv* and *spa* by Hochman (1971; Fig. 3B). However, $l(4)spa^{20}$ complements these two lethals but displays, when combined with spa^{pol}, an eye phenotype similar to that of spa^{pol} (Fig. 4D; Fu and Noll, 1997). In addition, $l(4)spa^{20}$ enhances the bristle phenotype of sv^n and sv^{de} in the eyes of transheterozygotes and causes a nearly complete loss of all shafts (Fig. 4A-C). Similarly, loss or reduction of shafts in macro- and microchaetae of the remaining body parts is more severe in transheterozygous combinations of sv^n or sv^{de} with $l(4)spa^{20}$ than in homozygous sv^n or sv^{de} flies (Fig. 4G-J). In particular, where shafts are completely lost in transheterozygotes, a second socket cell appears, mostly at macrochaetae and less frequently at microchaetae (Fig. 4I,J and unpublished results), which suggests that shaft cells are transformed into socket cells in the absence of *sv* function. Moreover, if Spa protein is indeed also the product of the *sv* gene, the consclusion that *sv* is required for the specification of shaft as opposed to socket cells would be consistent with our observation that Spa is expressed in shaft cells but absent from socket cells (Fig. 2A). However, these results so far only confirmed that *sv* and *spa* are neighboring mutations either of the same or of different genes.

Rescue of both *spa* and *sv* mutant phenotypes by *spa* transgenes

While analysis of the molecular nature of the four *spa-sv* alleles confirmed that $l(4)spa^{30}$, $l(4)spa^{47}$ and $l(4)spa^{66}$ are large deficiencies deleting the transcribed region of *spa*, it further revealed that $l(4)spa^{20}$ is a deletion as well. Isolation of the breakpoint of this deficiency $Df(4)spa^{20}$ from a genomic library showed that it deleted at least 13 kb of the upstream region of *spa* (Fig. 3A). Therefore, we tried to decide whether *sv* and *spa* are different genes or alleles of the same gene by attempts to rescue both *spa* and *sv* mutant phenotypes with transgenes in which the coding and promoter regions of *spa* were combined with the *spa* enhancer or a putative *sv* enhancer. When a 0.93 kb *Spe*I fragment derived from the 5′ end of the 4th intron of *spa* was used as enhancer to drive the expression of Spa in cone and primary pigment cells of homozygous spa^{pol} mutants, their mutant eye phenotype was completely rescued (Fig. 4E) as previously reported (Fu and Noll, 1997). Assuming that the sv^{de} and sv^n insertions, which mapped 1.6 kb and 1.8 kb upstream of the *spa* transcriptional start site (Fig. 3A), had occurred in, or close to, an enhancer regulating *spa* expression in bristle cells, we tested two different upstream fragments in combination with a *spa*-cDNA for rescue of sv^n mutants (see Materials and methods). Indeed, when expression of the *spa* transgene was controlled by a 6.7 kb upstream fragment of *spa*, the bristle phenotype of homozygous sv^n and of $sv^n/Df(4)spa^{20}$ mutants was rescued completely (not shown). By contrast, a

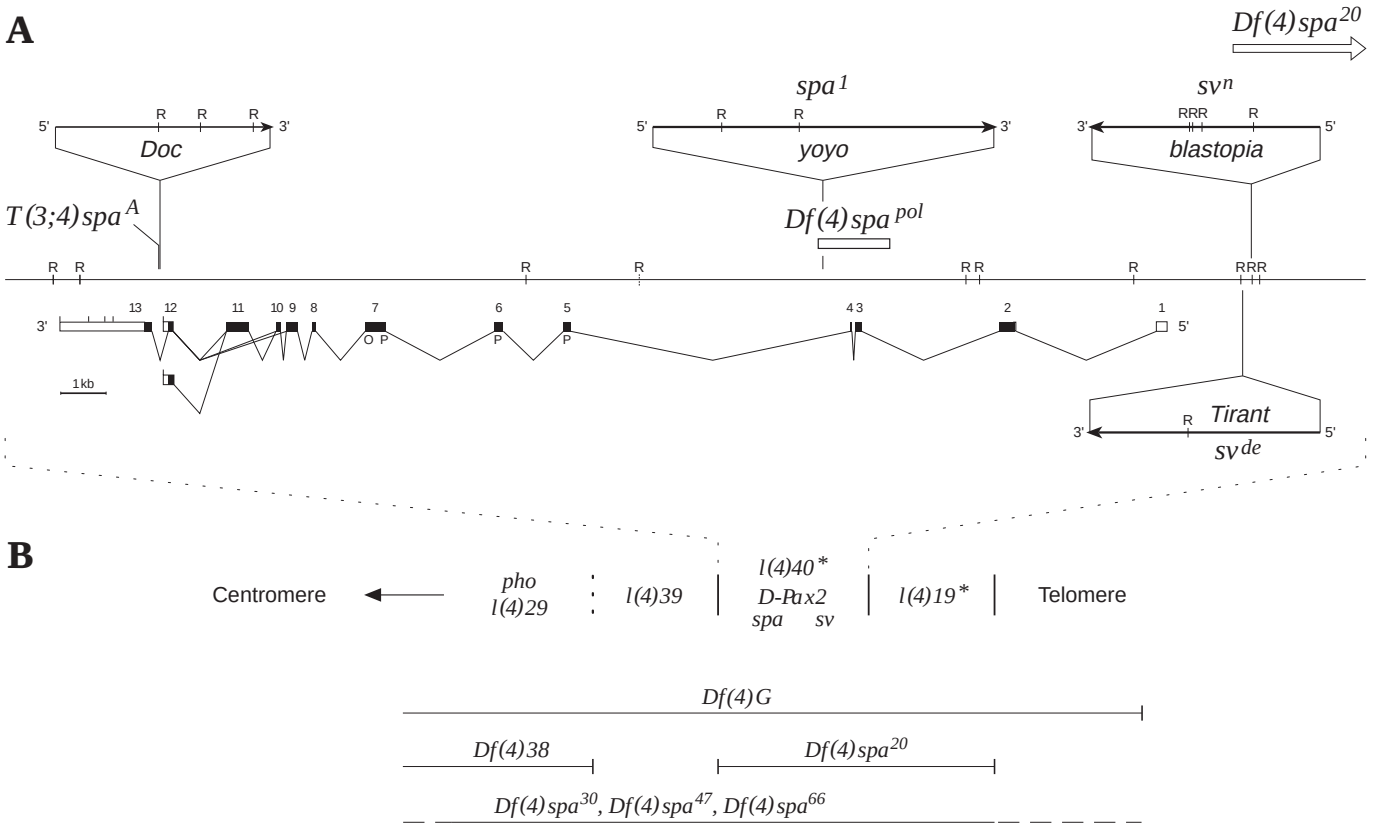

Fig. 3. Molecular and genetic map of the *Drosophila Pax2* (*D-Pax2*) locus. (A) Molecular lesions of *spa* and *sv* alleles of the *D-Pax2* locus. Mutations of *spa* and *sv* alleles and a polymorphism have been mapped with respect to genomic *Eco*RI sites (R) and the *D-Pax2* transcription unit. The mutations are a 1.58-kb deficiency extending from the second to the fourth intron (*Df(4)spa^{pol}*), an insertion of a 7.5 kb *yoyo* retrotransposon in the fourth intron (*spa^{1}*), or insertions of a 5.0 kb *blastopia* (*sv^{n}* which is identical to *sv^{35a}*) or of a defective 5.1 kb *Tirant* retrotransposon (*sv^{de}*) in the upstream region of *D-Pax2*. *Df(4)spa^{20}* is a deletion of at least 13 kb with its proximal breakpoint 1.4 kb upstream of the transcriptional start site of *D-Pax2*. *T(3;4)spa^{A}* marks the breakpoint of the reciprocal translocation *T(3;4)Ubx^{A} spa^{A}* which is located in the last intron of a polymorphic *D-Pax2* allele that carries in its last intron an insertion of a 4.73 kb *Doc* retroposon (O'Hare et al., 1991) and by itself has no effect on the phenotype (Fu and Noll, 1997). Below the *Eco*RI restriction map, the 13 exons and the open reading frame (in black; with paired domain P and octapeptide O) corresponding to the longest *D-Pax2* cDNAs are depicted, and differential splice variants are indicated (Fu and Noll, 1997). (B) Genetic map of the right telomeric region of the 4th chromosome. The orientation of *D-Pax2* in A is shown with respect to the most distal complementation groups on the right arm of the 4th chromosome that were mapped by Hochman (1971). In addition to *Df(4)G* and *Df(4)38* (Hochman, 1971), four deficiencies, *Df(4)spa^{20}*, *Df(4)spa^{30}*, *Df(4)spa^{47}* and *Df(4)spa^{66}*, obtained in this study are mapped. Three of them do not complement with any of the complementation groups shown and may extend on either side (dotted line) whereas *Df(4)spa^{20}* complements only with complementation groups proximal to *D-Pax2*. The asterisks in *l(4)19* and *l(4)40* indicate that all alleles of these mutants have been lost (Lindsley and Zimm, 1992). Hence, instead of *l(4)19*, a lethal allele of a locus distal to, and complementing, *sv* and *spa* (generously supplied by J. Kavaler and J. Posakony) was used for mapping. The orientation of the *D-Pax2* transcription unit was determined by in situ hybridization to *T(3;4)Ubx^{A} spa^{A}* polytene chromosomes with *D-Pax2* fragments located on either side of the *spa^{A}* breakpoint. *Df(4)spa^{20}* does not include the telomere since hybridization of a DNA fragment located 9 to 14 kb distal to *Df(4)spa^{20}* is barely resolved from a probe located 2 to 6 kb downstream from the 3′ end of *D-Pax2*. The dashed line between *l(4)29*, later called *pleiohomeotic* (*pho*), and *l(4)39* indicates that their relative positions are unclear and thus could be reversed (Hochman, 1971).

shorter 2.1 kb upstream region was able to rescue the bristle phenotype of *sv^{n}* mutants entirely on the notum and partially in the eye but not on the abdomen (not shown).

Since these results did not exclude the possibility that the partial rescue of *sv^{n}* was attributable to a gene located largely within the 2.1 kb upstream region of, but different from, *spa*, we attempted to rescue both the *spa^{pol}* and the *sv^{n}* phenotypes by a *Hs-spa* transgene in which expression of a *spa*-cDNA was under the control of the *hsp70* heat-shock promoter (see Materials and methods). Such a transgene was able to rescue the eye phenotype of *spa^{pol}* mutants to a considerable extent (Fig. 4F), as well as the bristle phenotype of *sv^{n}* mutants completely on the notum (Fig. 4K) and partially in the eye (Fig. 4L).

shaven and *sparkling* are enhancer mutations of the same gene

Nevertheless, these results were still consistent with the formal possibility that the 2.1 kb upstream region of *spa* included the entire coding region of *sv* whose product acted mainly, if not entirely, through the transcriptional activation of *spa*. If true, it should be possible to rescue *sv* mutants with a transgene that did not include the *spa* coding region. Therefore, we tried to rescue *sv^{n}* mutants by a transgene that consisted only of the 6.7

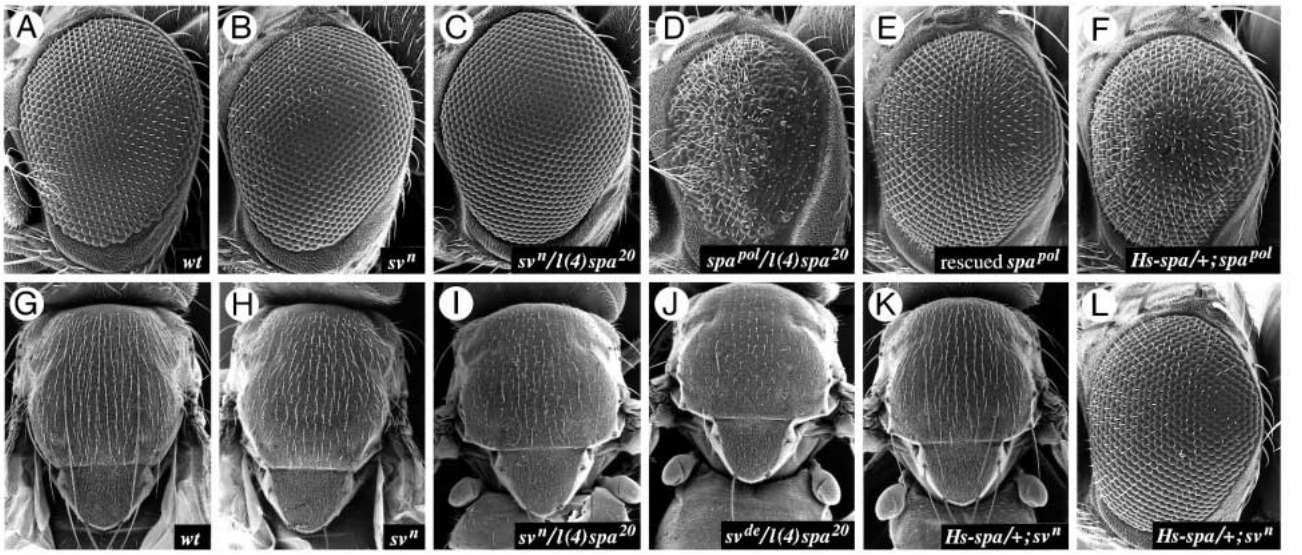

Fig. 4. Rescue of *sv* and *spa* phenotypes in the adult eye and notum by *D-Pax2* transgenes. Scanning electron micrographs of left eyes (A-F,L) or nota (G-K) of wild-type (A,G), homozygous sv^n (B,H), $sv^n/l(4)spa^{20}$ (C,I), $spa^{pol}/l(4)spa^{20}$ (D) and $sv^{de}/l(4)spa^{20}$ (J) flies are compared to those of homozygous spa^{pol} or sv^n flies whose mutant phenotypes have been completely (E) or partially (F,K,L) rescued by a *spa* transgene under control of the *hsp70* promoter (F,K,L) or of its own promoter and eye-specific enhancer (E) (cf. Materials and methods). The mutant phenotype of sv^{de} flies (not shown) is more extreme than that of sv^n flies except in the eye where both show a strong reduction in the number of eye bristles (B). In particular, shafts of bristles (microchaetae) are more strongly reduced in sv^{de} than in sv^n flies whereas large bristles (macrochaetae) are less affected in sv^{de} than in sv^n flies where shafts are strongly reduced in size or missing and occasionally transformed into sockets (H). In contrast to sv^n, sv^{de} flies are not fully viable and sterile (Lindsley and Zimm, 1992). $l(4)spa^{20}$ strongly enhances the phenotype of both sv^n (C,I) and sv^{de} (J) and behaves like *Df(4)G*. In A-F and L the dorsal side is up while in G-K anterior is up.

kb upstream region of *spa*, which, when combined with the *spa* coding region, had rescued sv^n mutants completely (see Materials and methods). As none of 22 such independent transgenic lines showed any rescue of sv^n, it follows that this region does not encode a transactivator but rather a *cis*-regulatory region of *spa* and that *sv* and *spa* are alleles of the same gene that have been mutated in different tissue-specific enhancers. Since this gene is the *Drosophila* homolog of the mouse *Pax2* gene (Fu and Noll, 1997), we call it *D-Pax2*.

Expression and genetic behavior of the $Df(4)spa^{20}$ allele

It is surprising that $Df(4)spa^{20}$, in which only the distal upstream region of *spa* is removed (Fig. 3A), does not complement spa^{pol} since none of the sequences required for the rescue of this mutant (Fig. 4E) is deleted. In agreement with this observation, no Spa protein was detectable in nuclei of cone and primary pigment cells in $Df(4)spa^{20}/spa^{pol}$ larval (Fig. 1D) and pupal discs (not shown). Consequently, *D-Pax2* is not expressed from the $Df(4)spa^{20}$ chromosome in these cells despite the presence of intact promoter, enhancer and transcribed regions. A possible explanation for this repression of *D-Pax2* is that the gene is subjected to a silencer that is moved to its vicinity as a result of the deficiency. Alternatively, since the deleted upstream region of *D-Pax2* extends towards the telomere (Fig. 3), the deficiency might permit telomeric heterochromatin to propagate into *spa* and repress it in cone and primary pigment cells.

Similarly, *D-Pax2* is not expressed from the $Df(4)spa^{20}$ chromosome in bristle cells of $Df(4)spa^{20}/sv^n$ mid-pupal eye discs (not shown), in which expression of sv^n is restricted to cone and primary pigment cells (Fig. 2C). At an earlier pupal stage, i.e., 24 hours after puparium formation at 25°C, levels of D-Pax2 protein are already reduced in shaft and perhaps also in glial cells of homozygous sv^n as compared to wild-type eye discs (not shown). Since the bristle phenotype of $Df(4)spa^{20}/sv^n$ flies is much stronger than that of sv^n mutants (Fig. 4B,C), we assume that D-Pax2 levels in $Df(4)spa^{20}/sv^n$ are even lower than in sv^n discs, which would suggest that *D-Pax2* is repressed on the $Df(4)spa^{20}$ chromosome of eye bristle cells already at early pupal stages. *D-Pax2* is also silenced on the $Df(4)spa^{20}$ chromosome in antennal discs since D-Pax2 levels in antennal discs (Fig. 1A) of spa^{pol} homozygotes seem wild-type (not shown) but are clearly reduced in $spa^{pol}/Df(4)spa^{20}$ mutants (Fig. 1D).

Whereas *D-Pax2* on the $Df(4)spa^{20}$ chromosome is completely repressed in the eye disc and possibly in other discs as well, it remains expressed in the PNS and CNS of homozygous $Df(4)spa^{20}$ embryos (Fig. 1F), although at levels that are considerably reduced compared to those of $Df(4)spa^{20}/spa^{pol}$ embryos (Fig. 1E) in which expression of spa^{pol} is similar, if not identical, to wild-type. The reduced embryonic expression of *D-Pax2* on the $Df(4)spa^{20}$ chromosome may result from a partial deletion of its embryonic enhancer(s) or from the two possible silencing mechanisms discussed above.

DISCUSSION

A gene may serve several distinct functions if its product is required repeatedly in the same or in different developmental

pathways. Such multipurpose genes (Lawrence, 1992) arise during evolution by a gene's acquisition of multiple enhancers that activate transcription in response to specific combinations of transcription factors at particular times and locations during development. The function of a gene thus largely depends on the specific time and location of its expression, i.e., on the specific enhancers by which its transcription is activated (Li and Noll, 1994; Xue and Noll, 1996). Here we have shown that the complementing mutant alleles *spa* and *sv* have lesions in separate enhancers of the same gene, *D-Pax2*, and thus reflect different functions of the same gene product, depending on its expression either in cone and primary pigment cells of the eye disc (Fu and Noll, 1997) or in bristle cells of developing adult mechanosensory organs. Our results bring into focus the problems arising from the conventional definition of a gene which fails to distinguish between its independently mutating coding and control regions.

Is *D-Pax2* a lethal gene?

Since a deficiency for *D-Pax2*, *Df(4)G* (Fig. 3B), was known to be fully viable over spa^{pol} and to display a clear rough eye phenotype, we expected to isolate null alleles of *D-Pax2* in a simple F_1 mutagenesis screen for mutants that fail to complement spa^{pol}. Astonishingly, we did not find any *bona fide* null alleles of *D-Pax2* that did not affect neighboring lethal genes as well. In fact, all *spa* mutant alleles that were obtained and failed to complement *sv* turned out to be rather large deficiencies that uncovered, in addition to *D-Pax2*, at least one neighboring lethal gene (Fig. 3B). Although large deficiencies generated by EMS mutagenesis have been observed previously, as, for example, in a screen for *Krüppel* (*Kr*) alleles (Preiss et al., 1985), their incidence was much lower than observed here. Nevertheless, it may be relevant that both *Kr* and *D-Pax2* are located relatively close to the telomere which might enhance the probability of generating large deficiencies by EMS treatment. An explanation for our failure to detect null alleles of *D-Pax2* that are not deficiencies might be that such null alleles, e.g., nonsense or frameshift mutations, show only a weak phenotype over spa^{pol} because of transvection, i.e., the transcription of the spa^{pol} gene might be activated in trans by the enhancer of the point mutant allele.

Since all null alleles of *D-Pax2* were deficiencies that also uncovered neighboring lethal loci, we do not know if *D-Pax2* itself is a lethal gene. Although Hochman (1971) reported the isolation of a lethal EMS-induced allele, *l(4)40*, which failed to complement both *sv* and *spa*, it has been lost before a molecular analysis was possible (Fig. 3B). Hence, we cannot rule out that it too was a deficiency uncovering a neighboring lethal locus although it complemented all other known neighboring lethals (Hochman, 1971). However that might be, it seems probable that *D-Pax2* is a lethal locus because *Df(4)G* or $Df(4)spa^{20}/sv^{de}$ flies are subviable and die shortly after eclosure even though sv^{de} is not a null allele and does not affect, for example, embryonic expression in the PNS and CNS. In addition, the fully viable dominant allele spa^{A} (Lindsley and Zimm, 1992), which is caused by a reciprocal translocation between *D-Pax2* and *Ubx* (Fig. 3A; see Materials and methods), is completely lethal over the deficiencies *Df(4)G* or $Df(4)spa^{20}$.

Role of *D-Pax2* in bristle development

We have previously demonstrated that Spa protein plays an important role in the development of the eye disc where it is required for differentiation of cone and primary pigment cells (Fu and Noll, 1997). Here we show that this is not the only function of Spa in the pupal retina but that Spa is also essential for differentiation of bristles. All bristles or mechanosensory organs of the adult fly arise in a simple stereotyped manner by three consecutive asymmetric divisions during which a single SOP gives rise to a neuron and three support cells (for review, see Posakony, 1994). The first division generates different siblings, one of which by subsequent division produces the cells that form the socket (tormogen) and shaft (trichogen), the other, the neuron and glial cell (thecogen). D-Pax2 or Sv appears to be required at various stages during bristle development. Initially, it is observed in SOPs and all four bristle precursor cells (Fu and Noll, 1997; and unpublished results). However, by mid-pupal stages it is no longer expressed in the tormogen and neuron, but continues to be expressed in the thecogen and is strongly elevated in the trichogen (Fig. 2A). If thecogen and trichogen, in response to opposite Notch (N) signaling, arise from their precursors by analogous asymmetric divisions, as proposed in an elegant model (Posakony, 1994), D-Pax2 expression must also react to N signaling in these cells in opposite ways. For example, while N signaling may activate *sv* or *D-Pax2* through Su(H) in glial cells, it may repress *D-Pax2* in socket cells.

Comparison of Spa protein levels in developing eye bristles of sv^{n} mutants with those of wild type (Fig. 2A,C) suggests that its expression needs to be maintained at least to mid-pupal stages for proper differentiation of trichogens and the formation of a shaft of normal size. However, trichogen cells are able to differentiate to some extent in hypomorphic sv^{n} mutants which express Spa during early pupal stages. If the levels of Spa are further reduced, for example in $sv^{n}/Df(4)G$ flies, the shafts are also shortened or completely missing. In that case, the shaft is replaced by a second socket, which indicates that the trichogen was no longer able to differentiate properly but became a tormogen. It appears therefore that D-Pax2 protein is not only required during differentiation of a shaft cell, but also serves at an earlier time to specify the fate of shaft versus socket cell. This would be consistent with *D-Pax2* being a target of the N signaling pathway.

The roles that *D-Pax2* plays in developing cone and primary pigment cells or in bristle cells, which manifest themselves in the various enhancer mutant alleles of *spa* and *sv* (Fig. 3A), are probably not the only functions of *D-Pax2* as evident from several additional locations where we were able to detect expression of Spa or D-Pax2 protein during embryonic and larval development. The expression of *D-Pax2* in the embryonic PNS may indicate that *D-Pax2* plays a function in the development of larval sense organs analogous to that of *sv* in the development of mechanosensory bristles of the adult.

Expression of D-Pax2 in the antennal disc may be homologous to Pax2 expression in the developing inner ear of mammals

Expression of D-Pax2 in the antennal disc is particularly interesting because it might reflect the conservation of another gene network in which *Drosophila* and vertebrate *Pax2*

participate and thus lends further support to the proposed conservation of gene networks (for a review, see Noll, 1993). The expression patterns of D-Pax2 protein in the antennal disc (Fig. 1A), as well as in the leg and wing disc (Fig. 1B,C), include expression in precursors of chordotonal organs, as judged by their striking resemblance to those of *atonal* (*ato*) (Jarman et al., 1993) and, more convincingly, by their absence in *ato* discs (Y. Shi and M. N., unpublished results). The chordotonal organs of the second antennal segment form the fly's auditory device, the so-called Johnston's organ (Ewing, 1978). Similar to *D-Pax2* in the development of Johnston's organ, *Pax2* expression begins very early during development of the mouse inner ear, in the otic placode, and differentiation of the auditory portion of the inner ear, the cochlea and cochlear ganglion, is completely blocked in *Pax2* null mutants (Torres et al., 1996). A hearing defect observed in human patients heterozygous for a gene encoding a truncated PAX2 protein suggests a very similar role for *PAX2* in man (Sanyanusin et al., 1995). Although it remains to be shown if *D-Pax2* plays a similarly decisive role in the development of Johnston's organ, the ear of the fly, it is an attractive hypothesis that the funtions of vertebrate and invertebrate *Pax2* have beeen conserved in the development of both the eye (Fu and Noll, 1997) and the ear.

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REFERENCES

Basler, K., Christen, B. and Hafen, E. (1991). Ligand-independent activation of the sevenless receptor tyrosine kinase changes the fate of cells in the developing *Drosophila* eye. *Cell* **64,** 1069-1081.

Bender, W., Akam, M., Karch, F., Beachy, P. A., Peifer, M., Spierer, P., Lewis, E. B. and Hogness, D. S. (1983). Molecular genetics of the bithorax complex in *Drosophila melanogaster*. *Science* **221,** 23-29.

Carroll, S. B. (1995). Homeotic genes and the evolution of arthropods and chordates. *Nature* **376,** 479-485.

Czerny, T., Bouchard, M., Kozmik, Z. and Busslinger, M. (1997). The characterization of novel *Pax* genes in the sea urchin and *Drosophila* reveal an ancient evolutionary origin of the *Pax2/5/8* subfamily. *Mech. Dev.* **67,** 179-192.

Ewing, A. W. (1978). The antenna of *Drosophila* as a 'love song' receptor. *Physiol. Entomol.* **3,** 33-36.

Frei, E., Baumgartner, S., Edström, J.-E. and Noll, M. (1985). Cloning of the *extra sex combs* gene of *Drosophila* and its identification by P-element-mediated gene transfer. *EMBO J.* **4,** 979-987.

Frommer, G., Schuh, R. and Jäckle, H. (1994). Localized expression of a novel micropia-like element in the blastoderm of *Drosophila melanogaster* is dependent on the anterior morphogen *bicoid*. *Chromosoma* **103,** 82-89.

Fu, W. and Noll, M. (1997). The *Pax2* homolog *sparkling* is required for development of cone and pigment cells in the *Drosophila* eye. *Genes Dev.* **11,** 2066-2078.

Garrell, J. and Modolell, J. (1990). The *Drosophila extramacrochaetae* locus, an antagonist of proneural genes that, like these genes, encodes a helix-loop-helix protein. *Cell* **61,** 39-48.

Hochman, B. (1971). Analysis of chromosome 4 in *Drosophila melanogaster*. II: Ethyl methanesulfonate induced lethals. *Genetics* **67,** 235-252.

Jarman, A. P., Grau, Y., Jan, L. Y. and Jan, Y. N. (1993). *atonal* is a proneural gene that directs chordotonal organ formation in the *Drosophila* peripheral nervous system. *Cell* **73,** 1307-1321.

Klemenz, R., Weber, U. and Gehring, W. J. (1987). The *white* gene as a marker in a new P-element vector for gene transfer in *Drosophila*. *Nucl. Acids Res.* **15,** 3947-3959.

Lawrence, P. A. (1992). *The Making of a Fly*. Oxford: Blackwell Scientific Publications.

Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* **276,** 565-570.

Lewis, E. B. and Bacher, F. (1968). Method of feeding ethyl methane sulfonate (EMS) to *Drosophila* males. *Drosophila Inform. Serv.* **43,** 193.

Li, X. and Noll, M. (1994). Evolution of distinct developmental functions of three *Drosophila* genes by acquisition of different cis-regulatory regions. *Nature* **367,** 83-87.

Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of* Drosophila melanogaster. San Diego, CA: Academic Press.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Moltó, M. D., Paricio, N., López-Preciado, M. A., Semeshin, V. F. and Martínez-Sebastián, M. J. (1996). *Tirant*: a new retrotransposon-like element in *Drosophila melanogaster*. *J. Mol. Evol.* **42,** 369-375.

Noll, M. (1993). Evolution and role of Pax genes. *Curr. Opin. Genet. Dev.* **3,** 595-605.

O'Hare, K., Alley, M. R. K., Cullingford, T. E., Driver, A. and Sanderson, M. J. (1991). DNA sequence of the Doc retroposon in the *white-one* mutant of *Drosophila melanogaster* and of secondary insertions in the phenotypically altered derivatives *white-honey* and *white-eosin*. *Mol. Gen. Genet.* **225,** 17-24.

Peifer, M., Karch, F. and Bender, W. (1987). The bithorax complex: control of segmental identity. *Genes Dev.* **1,** 891-898.

Posakony, J. W. (1994). Nature versus nurture: asymmetric cell divisions in *Drosophila* bristle development. *Cell* **76,** 415-418.

Preiss, A., Rosenberg, U. B., Kienlin, A., Seifert, E. and Jäckle, H. (1985). Molecular genetics of *Krüppel*, a gene required for segmentation of the *Drosophila* embryo. *Nature* **313,** 27-32.

Sánchez-Herrero, E., Vernós, I., Marco, R. and Morata, G. (1985). Genetic organization of *Drosophila* bithorax complex. *Nature* **313,** 108-113.

Sanyanusin, P., Schimmenti, L. A., McNoe, L. A., Ward, T. A., Pierpont, M. E. M., Sullivan, M. J., Dobyns, W. B. and Eccles, M. R. (1995). Mutation of the *PAX2* gene in a family with optic nerve colobomas, renal anomalies and vesicoureteral reflux. *Nature Genet.* **9,** 358-364.

Thomas, U., Jönsson, F., Speicher, S. A. and Knust, E. (1995). Phenotypic and molecular characterization of Ser^{D}, a dominant allele of the *Drosophila* gene *Serrate*. *Genetics* **139,** 203-213.

Torres, M, Gómez-Pardo, E. and Gruss, P. (1996). *Pax2* contributes to inner ear patterning and optic nerve trajectory. *Development* **122,** 3381-3391.

Viggiano, L., Caggese, C., Barsanti, P. and Caizzi, R. (1997). Cloning and characterization of a copy of *Tirant* transposable element in *Drosophila melanogaster*. *Gene* **197,** 29-35.

Xue, L. and Noll, M. (1996). The functional conservation of proteins in evolutionary alleles and the dominant role of enhancers in evolution. *EMBO J.* **15,** 3722-3731.

Zipursky, S. L., Venkatesh, T. R., Teplow, D. B. and Benzer, S. (1984). Neuronal development in the *Drosophila* retina: monoclonal antibodies as molecular probes. *Cell* **36,** 15-26.