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

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Accepted 28 May; published on WWW 9 July 1998

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\textsuperscript{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\textsuperscript{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\textsuperscript{20}/cyD spa\textsuperscript{pol} and T(3;4)Ubx\textsuperscript{A}, cu kar Ubx\textsuperscript{A} spa\textsuperscript{A}/MKRS stocks in the λ-DASH II vector (Stratagene) to isolate phages harboring the Df(4)spa\textsuperscript{20} and the T(3;4)Ubx\textsuperscript{A} spa\textsuperscript{A} breakpoints, respectively. Mutant DNA fragments of sv\textsuperscript{n}, sv\textsuperscript{35a} and sv\textsuperscript{de} were isolated from genomic DNA of sv\textsuperscript{n}/sv\textsuperscript{n}, sv\textsuperscript{35a}/sv\textsuperscript{35a}, sv\textsuperscript{de}/cyD stocks by PCR, by means of primers in the first intron (0.34 kb downstream of the first exon) of D-Pax2 (5′-TGTTACGAGGTCA TTGGAGCTT-3′ and 2.3 kb upstream of its transcriptional start site (5′-GTGCATCGACTTGTTAAAGCC-3′). That the enlarged PCR fragment obtained from sv\textsuperscript{de}/cyD DNA originated from the sv\textsuperscript{de} rather than from the cyD chromosome was verified by PCR of sv\textsuperscript{de}/Df(4)spa\textsuperscript{20} and sv\textsuperscript{de}/Df(4)spa\textsuperscript{A}. The
breakpoints of Df(4)spa\(^{20}\) and T(3;4)Ubx\(^{3}\)spa\(^{3}\) and sites of insertion in sv\(^{v}\), sv\(^{55a}\), 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\(^{v}\) and sv\(^{55a}\) stocks had identical insertions of the Spa 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\(^{55a}\), 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; Moltö 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 subclass 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\(^{l}\) 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 retrotransposon (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\(^{3}\) spa\(^{5}\), is a complicated rearrangement that includes several additional inversions on the third, but none on the fourth, chromosome (Lindsay 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 retrotransposon; 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\(^{l}\)(S(Ubx\(^{3}\))’)/spa\(^{5}\)(S(Ubx\(^{3}\))’) fragment within the sequence 5’-ACTAGTCTAGGACA | GCGTCTAGCTCCGG-3’ at coordinate 314581 of the BX-C sequence and the breakpoint of the reciprocal spa\(^{3}\)(Ubx\(^{3}\))/spa\(^{l}\)(Ubx\(^{3}\))’ fragment within the sequence 5’-ACTGCAAGATAATGC | AGGCCGA | TAATGTGTATTTAGA-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\(^{3}\)(Ubx\(^{3}\))/spa\(^{l}\)(Ubx\(^{3}\))’ 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 SpeI/spa-PCG2 harboring a spa transgene regulated by a 926-bp SpeI enhancer fragment of intron 4 (Fu and Noll, 1997).

For rescue of sv\(^{v}\) and spa\(^{pol}\) mutants, a Hs-spa construct was prepared by cloning a 2.8 kb EcoRI-XhoI fragment of cpx1 spa- cDNA with which 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 KpnI and EcoRI 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 Xhol-KpnI 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 Xhol-KpnI 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 XhoI site (5’-CTAGTCTAGCTCCAGGCGAATTCTACAA-3’) and of a leader primer with an attached KpnI site (5’-GGGTTAATCTTGTGCTAAATATCCAGAA-3’). The 6.7-spa construct was generated by replacing the 926-bp SpeI enhancer fragment and the 330 bp spa promoter of the SpeI/spa-PCG2 construct by an XhoI-BsrWI fragment that extends 6.7 kb upstream from the transcriptional start site located a few bp upstream of the BsrWI site. As a control, the 6.7 kb XhoI-BsrWI 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\(^{v}\) 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\(^{v}\) embryos. Of all constructs several independent transgenic lines were obtained except for Hs-spa of which only one healthy line (w\(^{1118}\); 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 eclosion. Only slight rescue of the spa\(^{pol}\) phenotype was observed without temperature shift. Hs-spa+/; sv\(^{v}\) flies displayed complete rescue of the sv\(^{v}\) 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\(^{v}\) 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 eclosion (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\(^{38}\), spa\(^{21}\) and spa\(^{de}\), were generated, all embryonic lethal deficiencies.

**Fly stocks**

Mutant stocks of Df(4)F;ci\(^{D}\), ci\(^{D}\) spa\(^{pol}\)/ey\(^{D}\), spa\(^{pol}\)/spa\(^{pol}\), sv\(^{v}\)/sv\(^{v}\), sv\(^{55a}\)/sv\(^{55a}\), sv\(^{de}\)/ey\(^{D}\), l(4)29/ci\(^{D}\), l(4)39/ci\(^{D}\) and Df(4)38/ci\(^{D}\) were...
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<sup>pol</sup> 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<sup>a</sup> 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<sup>pol</sup> mutants, its expression in bristle cells appears unaffected (Fig. 2B). The situation is reversed in sv<sup>a</sup> 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<sup>a</sup> eye discs (not shown), in agreement with the observation that sv<sup>a</sup> flies display a hypomorphistic 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<sup>a</sup>, sv<sup>35a</sup> and sv<sup>de</sup>, 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 EcoRI fragment about 1.6 kb upstream from the transcriptional start site of spa (Fig. 3A). While sv<sup>a</sup> and sv<sup>35a</sup> turned out to be identical insertions of the 5.0 kb blastopita retrotransposon (Frommer et al., 1994), a 5.1 kb Tirant element (Garrell and Modolell, 1990) is inserted in sv<sup>de</sup>. 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<sup>20</sup> 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<sup>20</sup>/ci<sup>D</sup> spa<sup>pol</sup> (D,E) and homozygous l(4)spa<sup>20</sup> mutants (F). Only heterozygous offspring of the l(4)spa<sup>20</sup>/ci<sup>D</sup> spa<sup>pol</sup> stock survive to the larval stage of the disc shown in D since both ci<sup>D</sup> and l(4)spa<sup>20</sup> 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<sup>20</sup> (F) as compared to l(4)spa<sup>20</sup>/ci<sup>D</sup> spa<sup>pol</sup> embryos (E), and both expression patterns can be easily distinguished from the disturbed pattern of homozygous ci<sup>D</sup> spa<sup>pol</sup> embryos obtained from the same l(4)spa<sup>20</sup>/ci<sup>D</sup> spa<sup>pol</sup> 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.
et al., 1984).

Different types of bristle cells have been identified by position and in cone (c) and primary pigment cells (pr) and in two of the four imaginal discs of trichogens (tr) or shaft cells and of thecogens (th) or glial cells. In addition, l(4)spa20 enhances the bristle phenotype of svn and svde 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 svn or svde with l(4)spa20 than in homozygous svn or svde 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 conclusion 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.

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)spa20, l(4)spa20, l(4)spa47 and l(4)spa66, were obtained, of which none complemented svn. Three of these new spa alleles, l(4)spa20, l(4)spa47 and l(4)spa66, 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)spa20 complements these two lethals but displays, when combined with spepol, an eye phenotype similar to that of spepol (Fig. 4D; Fu and Noll, 1997). In addition, l(4)spa20 enhances the bristle phenotype of svn and svde 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 svn or svde with l(4)spa20 than in homozygous svn or svde 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 conclusion 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)spa20, l(4)spa47 and l(4)spa66 are large deficiencies deleting the transcribed region of spa, it further revealed that l(4)spa20 is a deletion as well. Isolation of the breakpoint of this deficiency Df(4)spa20 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 SpeI 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 spepol mutants, their mutant eye phenotype was completely rescued (Fig. 4E) as previously reported (Fu and Noll, 1997). Assuming that the svde and svn 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 svn 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 svn and of svn/Df(4)spa20 mutants was rescued completely (not shown). By contrast, a
shorter 2.1 kb upstream region was able to rescue the bristle phenotype of $sv^a$ 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 $sv^a$ 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 $sv^a$ 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 $sv^a$ 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 $sv^a$ mutants by a transgene that consisted only of the 6.7 kb upstream region of, and different from, $spa$. Hence, instead of $spa$, the transcriptional start site of $spa$ was determined by in situ hybridization to polytene chromosomes with differential splice variants are indicated (Fu and Noll, 1997). Below the EcoRI 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. Kavalier and J. Posakony) was used for mapping. The orientation of the $D-Pax2$ transcription unit was determined by in situ hybridization to $T(3;4)$-blastopia $spa^a$ 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 $sv$ was attributable to a gene located largely with respect to genomic EcoRI sites (R) and the $D-Pax2$ transcription unit.

**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 have been mapped with respect to genomic EcoRI sites (R) and the $D-Pax2$ transcription unit. The mutations are a 1.58-kb deficiency extending from the second to the fourth intron ($Df(4)spa^{30}$), an insertion of a 7.5 kb $yoyo$ retrotransposon in the fourth intron ($spa^l$), or insertions of a 5.0 kb blastopia ($sv^a$ 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 $T(3;4)$-blastopia $spa^a$ 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 $Hs$-$spa$ unit. The mutations are a 1.58-kb deficiency extending from the second to the fourth intron ($Df(4)spa^{30}$), an insertion of a 7.5 kb $yoyo$ retrotransposon in the fourth intron ($spa^l$), or insertions of a 5.0 kb blastopia ($sv^a$ 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 $T(3;4)$-blastopia $spa^a$ 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 $Doc$ retrotransposon (O’Hare et al., 1991) and by itself has no effect on the phenotype (Fu and Noll, 1997). Below the EcoRI 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. Kavalier and J. Posakony) was used for mapping. The orientation of the $D-Pax2$ transcription unit was determined by in situ hybridization to $T(3;4)$-blastopia $spa^a$ 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 $sv$ was attributable to a gene located largely with respect to genomic EcoRI sites (R) and the $D-Pax2$ transcription unit. The mutations are a 1.58-kb deficiency extending from the second to the fourth intron ($Df(4)spa^{30}$), an insertion of a 7.5 kb $yoyo$ retrotransposon in the fourth intron ($spa^l$), or insertions of a 5.0 kb blastopia ($sv^a$ 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 $T(3;4)$-blastopia $spa^a$ 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 $Doc$ retrotransposon (O’Hare et al., 1991) and by itself has no effect on the phenotype (Fu and Noll, 1997). Below the EcoRI 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 $D-Pax2$. 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or nota (G-K) of wild-type (A,G), homozygous svn (B,H), svn/l(4)spa20 (C,I), spa pol/l(4)spa20 (D) and svd/l(4)spa20 (J) flies are compared to those of homozygous spa pol or svn 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 svde flies (not shown) is more extreme than that of svn 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 less affected in svde than in svn flies whereas large bristles (macrochaetae) are more strongly reduced in svde than in svn flies whereas large bristles (macrochaetae) are less affected in svde than in svn flies where shafts are strongly reduced in size or missing and occasionally transformed into sockets (H). In contrast to svn, svde flies are not fully viable and sterile (Lindsley and Zimm, 1992). l(4)spa20 strongly enhances the phenotype of both svn (C,I) and svd (J) and behaves like Df(4)G. In A-F and L the dorsal side is up while in G-K anterior is up.

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 svn (B,H), svn/l(4)spa20 (C,I), spa pol/l(4)spa20 (D) and svd/l(4)spa20 (J) flies are compared to those of homozygous spa pol or svn 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 svde flies (not shown) is more extreme than that of svn 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 less affected in svde than in svn flies whereas large bristles (macrochaetae) are more strongly reduced in svde than in svn flies whereas large bristles (macrochaetae) are less affected in svde than in svn flies where shafts are strongly reduced in size or missing and occasionally transformed into sockets (H). In contrast to svn, svde flies are not fully viable and sterile (Lindsley and Zimm, 1992). l(4)spa20 strongly enhances the phenotype of both svn (C,I) and svd (J) and behaves like Df(4)G. In A-F and L the dorsal side is up while in G-K anterior is up.

Expression and genetic behavior of the Df(4)spa20 allele

It is surprising that Df(4)spa20, 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)spa20/spa pol larval (Fig. 1D) and pupal discs (not shown). Consequently, D-Pax2 is not expressed from the Df(4)spa20 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)spa20 chromosome in bristle cells of Df(4)spa20/svn mid-pupal eye discs (not shown), in which expression of svn 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 svn as compared to wild-type eye discs (not shown). Since the bristle phenotype of Df(4)spa20/svn flies is much stronger than that of svn mutants (Fig. 4B,C), we assume that D-Pax2 levels in Df(4)spa20/svn are even lower than in svn discs, which would suggest that D-Pax2 is repressed on the Df(4)spa20 chromosome of eye bristle cells already at early pupal stages. D-Pax2 is also silenced on the Df(4)spa20 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)spa20 mutants (Fig. 1D).

Whereas D-Pax2 on the Df(4)spa20 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)spa20 embryos (Fig. 1F), although at levels that are considerably reduced compared to those of Df(4)spa20/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)spa20 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
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 F1 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 EMS 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, more importantly, D-Pax2 is a lethal locus because Df(4)G or Df(4)spa^20/sv^de flies are subviable and die shortly after eclosion 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^4 (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 (theogen). 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 theogen and is strongly elevated in the trichogen (Fig. 2A). If the theogen 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^de 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^de mutants which express Spa during early pupal stages. If the levels of Spa are further reduced, for example in sv^de/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 functions of vertebrate and invertebrate Pax2 have been conserved in the development of both the eye (Fu and Noll, 1997) and the ear.

We thank J. Kavaler and J. Posakony, A. Carpenter and the Drosophila stock centers at Bloomington, Umea and Bowling Green for mutant stocks, as well as K. Basler for his pKB256 P-element vector. We are indebted to Urs Jauch (Institut für Pflanzenbiologie, University of Zurich) and Thomas Gutjahr for performing the scanning electron microscopy and to Fritz Ochsenbein for expert art work. We are also grateful to Werner Boll, Michael Daube and Thomas Müller for DNA sequencing and to all of our colleagues for numerous discussions. We thank J. Alcedo, K. Basler, E. Hafen, W. Boll and H. Noll for criticism and comments on the manuscript. This work has been supported by Swiss National Science Foundation grant 31-40874.94 (to M. N.) and by the Kanton Zürich.

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