To understand evolution at the molecular level, it is important to find out how the genetic program of an organism can be altered to generate new organisms fit to survive. Such knowledge would not only explain evolution, but also provide insight into alternative and future evolutionary pathways that have not or not yet occurred. A direct way of trying to alter the genetic program of a developing organism would be to express a transcription factor at a new point in time or space. Indeed, expression of a gene in new spatiotemporal patterns through the acquisition of additional or modified enhancers is probably a major evolutionary mechanism underlying functional diversification (Li and Noll, 1994; Carroll, 1995; Xue and Noll, 1996; Greer et al., 2000). Its immediate consequence is a change in the genetic program of part of the developing organism, whereby an old developmental pathway is altered into a new one or replaced by one that was deployed in a different part of the organism. Paradigms for this latter case are the homeotic mutations, which are mostly deleterious for the organism but in rare cases may be advantageous during evolution (Lewis, 1978; Carroll, 1995).

How frequently are changes in the genetic program successful during evolution? An exhaustive answer to this question would allow us to assess the spectrum of possible organisms that could evolve. It is unclear whether a satisfactory answer to this exceedingly complex question can or ever will be found, particularly if the number of possible successful changes for any given organism is large. We have therefore tried to investigate the much simpler question of what are the consequences of expressing a transcription factor ectopically during development. To investigate how ectopic transcription factors affect a specific developmental pathway, we have examined their effects on Drosophila eye development when ectopic expression occurred at an early and at a late stage: before and after cell proliferation.

We show that probably most of these events are deleterious and hence generate organisms not fit to survive. In particular, we found that early ectopic expression of several transcription factors in the primordial eye-antennal disc interferes with the early function of Eyeless (Ey) and blocks cell division, thus generating headless flies, a phenotype much stronger than reported previously for ey mutants (Halder et al., 1998). This inhibition of the cell cycle can be reduced or relieved by D-CycE or D-Myc, which suggests a specific block during the G1 phase. Similarly, ectopic expression at a late developmental stage in cone cell precursors interferes with their differentiation. We propose that this developmental pathway interference is a general surveillance mechanism that eliminates most aberrations in the genetic program during development and evolution, and thus seriously restricts the pathways that evolution may take.

**SUMMARY**

Ectopic expression of transcription factors in eye-antennal discs of Drosophila strongly interferes with their developmental program. Early ectopic expression in embryonic discs interferes with the developmental pathway primed by Eyeless and generates headless flies, which suggests that Eyeless is necessary for initiating cell proliferation and development of both the eye and antennal disc. Interference occurs through a block in the cell cycle that for some ectopic transcription factors is overcome by D-CycE or D-Myc. Late ectopic expression in cone cell precursors interferes with their differentiation. We propose that this developmental pathway interference is a general surveillance mechanism that eliminates most aberrations in the genetic program during development and evolution, and thus seriously restricts the pathways that evolution may take.

Key words: Developmental pathway interference, eyeless, Headless, Evolution, Drosophila
cassette (from E. Hafen). *spa-Gal4* was prepared by cloning the 7.1 kb EcoRI genomic fragment of *D-Pax2* (Fu and Noll, 1997), extending from intron 2 into intron 4 and including the *spa* enhancer, into the NotI site of the pDA188.1 vector (a P element vector including the *hsp70* minimal promoter, the Gal4-coding region and the *tabulin1α* trailer, prepared and provided by D. Nellen and K. Basler). To construct *spa-Poxn*, a 1.73 kb fragment, extending from an EcoRI site introduced 23 bp upstream of the coding region of the P4c6 *poxn* cDNA to an artificial EcoRI site 70 bp downstream of the polyA addition site of *poxn*, was ligated downstream of 300 bp promoter region and 289 bp adjacent leader of the five tandem repeats of the region and 289 bp adjacent leader of the *spa* enhancer (Fu and Noll, 1997), the complete insert was transferred as 3.26 kb fragment from the P-element vector pW6 (Klemenz et al., 1987) to produce *spa-Poxn*.

*UAS-Poxn* was prepared by combining the EcoRI-BstXI 5′-fragment from pSK–PoxnPD (an EcoRI subclone encoding the paired domain of *Poxn*, obtained by PCR amplification of the P4c6 *poxn* cDNA with the primers 5′-CATCGAATTCATCCGACACAGT-CAA-3′ and 5′-GGCGAATTCATCAGGATGTCCTC-3′, in the blueScript cDNA library (Dambly-Chaudière et al., 1992) into the EcoRI site of pUAST (Brand and Perrimon, 1993). *UAS-Gsb*, *UAS-Prd* and *UAS-Poxn* were obtained by cloning the gsb-cDNA BSH9c2 (Baumgartner et al., 1987), the pRL-cDNA c7340.6 (Frigerio et al., 1986) and the 2.6 kb *poxn*-cDNA P29c1 (Bopp et al., 1989), respectively, into the EcoRI site of pUAST. *UAS-D-Pax2* was constructed by cloning the 2.8 kb EcoRI-XbaI fragment of the *D-Pax2* cDNA cpx1 (Fu and Noll, 1997) into pUAST. *UAS-Toy* was prepared by ligation into pUAST of the 2.1 kb EcoRI insert of a nearly fully-length toy-cDNA clone (10.4) isolated from an eye disc cDNA library in Agt10 (prepared by A. Kowman and provided by G. M. Rubin).

*UAS>*w>*D-Pax2* was constructed by first cloning the 2.8 kb EcoRI-XbaI fragment of the *D-Pax2*-cDNA cpx1 into the EcoRI site of pKB342 (a blueScript vector including the trailer of *tabulin1α*, provided by K. Basler). The insert, which consisted of the D-Pax2-cDNA and the *tabulin1α* trailer, was removed as a KpnI-XbaI fragment and cloned into pM51, a Carnegie 2-derived P-element vector (Rubin and Spradling, 1983) analogues to pUAST, including the five tandem repeats of the *UAS* sequence and the *hsp70* minimal promoter, but without the marker gene (provided by K. Basler). Finally, the mini-white marker gene was introduced as FRT cassette, isolated as KpnI fragment from pKB340 (pUC19 clone of direct FRT repeats flanking the *hsp70* trailer and mini-white; gene provided by K. Basler), into the KpnI site located between the *hsp70* promoter and the D-Pax2-cDNA.

*UAS-GE* was constructed by PCR mutagenesis. Two fragments overlapping in the region that included the three mutations (underlined in primers GE1 and GE2) altering the binding specificity of the Gsb paired domain to that of Ey were amplified by PCR using the two sets of primers: gspbдрbox5′ (5′-ACCAGGAATTCATGGCTTGTTCCGGCTTCC-3′) with GE2 (5′-AGAGACCGCACTTGTAGAGACTTTCGCCGGG3′) and GE1 (5′-CCGGATCCAGGCTTCTCTAATGGCCTGCTTCC-3′) with gspbдрbox3′ (5′-CCGGTCACTAGCCCGCAGTCTGTTGGA3′), gspb cDNA BSH9c2 was used as template. Subsequently, 1/50 of the volume of each PCR reaction were combined, amplified by PCR with the gspbдрbox5′ and gspbдрbox3′ primers, and inserted as EcoRI-SalI fragment into blueScript plK* for sequence verification. Finally, the 0.43 kb EcoRI-BamHI fragment of this insert was combined with the 1.05 kb BamHIEcoRI fragment from BSH9c2 in the EcoRI site of pUAST to generate *UAS-GE*.

The transgenes encoding wild-type and mutated Gsb proteins shown in Fig. 5 were constructed as follows. *UAS-Gsb*, *UAS-GsbΔP*, *UAS-GsbΔH*, *UAS-GsbΔN*, *UAS-GsbΔPoxn* and *UAS-GsbΔPoxnC* were prepared by removing the inserts from the corresponding pAR clones (Xue and Noll, 1996; Xue et al., 2001) by *XbaI* and *NheI* digestion and ligation in the proper orientation into the XbaI site of the pUAST vector (Brand and Perrimon, 1993). Note that this UAS-*Gsb* transgene, in contrast to the one described above, encodes no 5′ leader derived from gsb, but generates, if combined with *ey-Gal4*, the same spectrum of headless phenotypes (see Fig. 2A-E). *UAS-GsbP17L* was prepared by subcloning the EcoRI insert of pKSpL5-GsbP17L (Xue et al., 2001) into pGEM-2 in such an orientation that it could be removed as *XbaI*-NheI fragment for ligation into pUAST. Finally, to obtain *UAS-PoxnN+GsbC*, its EcoRI insert was first constructed in pKSpL5 (Xue and Noll, 1996) and subsequently cloned into pUAST. This was achieved by PCR amplification of a 400 bp EcoRI-blunt-end fragment, which encodes the N-terminal Poxn paired domain, with the use of the pSK–PoxnPD subclone as template and the primers 5′-AAAAACTTTCATAAAGGG-3′ (T3 primer) and 5′-GACCGTGGTCGGCAGAATC-3′ (poxnP-7 primer), and subsequent ligation with the 750 bp FspI-EcoRI fragment of the BSH9c2 *gsb*-cDNA (Baumgartner et al., 1987), which encodes the C-terminal moiety of Gsb, into the EcoRI site of pKSpL5. Several transgenic lines of each construct were obtained by P-element-mediated germline transformation according to standard procedures.

**Fly stocks**

The following fly stocks were used:

- spa*pol* (Fu and Noll, 1997),
- w; sev-Poxn/CyO,
- w; sev-PoxnTM3, Sb (Dambly-Chaudière et al., 1992),
- w; sev-PoxnTM3, Sb; spa*pol*,
- w; spa-Gal4,
- w; spa-Gal4; spa*pol*,
- w; sev-D-Pax2-4/TM3, Sb,
- w; spa-D-Pax2 (3rd chromosome; *D-Pax2* transgene whose expression is regulated by its own promoter and the *spa* enhancer, included in a 926 bp *spe* fragment of intron 4 of *D-Pax2*; Fu and Noll, 1997),
- w; spa-Poxn,
- w; spa-Poxn; spa*pol*,
- w; spa-Gal4 (3rd chromosome; from E. Hafen),
- w; UAS-Poxn-6 (2nd chromosome),
- w; UAS-Poxn-5 (3rd chromosome),
- w; ey-Gal4 (2nd chromosome; Hauck et al., 1999),
- w; UAS-D-Pax2-1 (3rd chromosome),
- w; UAS-Gsb-7 (2nd chromosome),
- w; UAS-Gsb-1 (3rd chromosome),
- w; UAS-Poxn (3rd chromosome),
- w; UAS-Prd-1 (3rd chromosome),
- w; UAS-Toy-6/TM3, Sb,
- y; w; UAS-Ey/TM3, Sb (Haldar et al., 1995),
- w; UAS-Gsb-7/CyO; UAS-Ey/TM3, Sb,
- y; w hs70-rop,
- w; ey-Gal4; UAS>*w>*D-Pax2-1,
- w; UAS-GE-8 (3rd chromosome),
- w; UAS>*w>*D-Pax2-2 (1st chromosome),
- w; UAS-Gsb-4 (3rd chromosome),
- w; UAS-GsbΔP-10,
- w; UAS-GsbΔH-8 (2nd chromosome),
- w; UAS-GsbN-3 (3rd chromosome),
- w; UAS-Gsb1 (3rd chromosome),
- w; UAS-GsbP17L-7 (3rd chromosome),
- w; UAS-GsbN+PoxnC-1 (2nd chromosome),
- w; UAS-PoxnN+GsbC-5 (2nd chromosome),
- w; UAS-Dac/TM3, Sb (Shen and Mardon, 1997),
- w; UAS-Ubx/TM3, Sb Ser,
- w; TM6, UAS-En/lethal,
- w; UAS-Ato/Cyo (Jarman et al., 1993),
- w; UAS-Mef2 (3rd chromosome; Lin et al., 1997),
- w; UAS-Sim (3rd chromosome; Xiao et al., 1996),
- w; UAS-D-Myc (3rd chromosome) (Johnston et al., 1999),
RESULTS

Ectopic expression of Poxn interferes with D-Pax2 functions in eye development

We first investigated the effect of ectopic expression of a transcription factor on a late stage of *Drosophila* eye development by examining the consequences of ectopic *pox neuro* (*poxn*) expression in cone cell precursors on their differentiation program. Ectopic expression of a *poxn* transgene under the control of a *sevenless* (*sev*) enhancer and hsp70 promoter, *sev-Poxn*, produces a dominant rough eye phenotype, which is presumably caused by the expression of Poxn in the *sev*-expressing subpopulation of ommatidial precursor cells (Dambly-Chaudière et al., 1992) consisting of the photoreceptors R3, R4, R7 and the four cone cells (Tomlinson et al., 1987). This *sev-Poxn* phenotype (Fig. 1A) resembles that of the *D-Pax2* mutant *spa pol* (Fig. 1B), in which transcription of *D-Pax2* appears to be abolished in third instar eye discs, particularly in cone cell precursors whose development, as a consequence, is severely disturbed (Fu and Noll, 1997; Fu et al., 1998). In the wild type, ommatidial expression of *D-Pax2* occurs first in cone cell precursors, whereas Poxn is never expressed in eye discs. Hence, expression of Poxn in cone cell precursors most probably inhibits the wild-type function of *D-Pax2* in these cells and thereby interferes with normal cone cell development. Consistent with this hypothesis, cobalt sulfide staining of *sev*-Poxn/+ mid-pupal eye discs revealed that arrangement, shape and number of cone cells were disturbed (not shown), as is typical for the *spa pol* phenotype (Fu and Noll, 1997). To test if the *sev-Poxn* phenotype results from the interference of Poxn expression with *D-Pax2* function in the eye disc, we examined if its severity depended on *D-Pax2* protein levels. When one copy of *D-Pax2* is substituted by a *spa pol* allele in *sev-Poxn/+* flies, the eye phenotype is stronger than that of *spa pol* mutants (Fig. 1B), lacks all lenses and most bristles, and closely resembles that of *lozenge* (*lz*) null mutants (Daga et al., 1996). Moreover, much fewer cone cells are present in *sev-Poxn/+; spa pol* than in *spa pol* mid-pupal eye discs and those observed are smaller and seem to be in the process of apoptosis (not shown). Finally, a single copy of *sev-D-Pax2* or *spa-D-Pax2* rescues the *sev-Poxn* phenotype to wild type (Fig. 1E,F), an observation corroborated by histological sections (not shown). We conclude that ectopic expression of Poxn under the control of the *sev* enhancer interferes only with functions in the developing eye that *D-Pax2* can provide when expressed under the control of the *spa* enhancer. Nevertheless, the eye phenotype of *spa pol*, caused by a complete loss of ommatidial *D-Pax2* transcription, is considerably enhanced by *sev-Poxn* (Fig. 1D). It follows that Poxn interferes not only with functions of *D-Pax2* but also with functions of other genes, normally provided by *D-Pax2* as well.
The ability of Poxn to interfere with D-Pax2 functions crucially depends on absolute and relative levels of Poxn and D-Pax2 and, equally importantly, on the time of ectopic Poxn expression. This follows from a series of experiments in which Poxn was expressed under the control of the spa enhancer (Fu et al., 1998; Flores et al., 2000), which acts later than svb but with similar strength. This regulation of ectopic poxn transcription was either direct, as in the case of spa-Poxn and sev-Poxn transgenes, or indirect when amplified to produce higher levels of ectopic Poxn by the use of the Gal4/UAS system. Thus, sev-Poxn/+ flies display a much stronger rough eye phenotype (Fig. 1A) than spa-Gal4/+; UAS-Poxn/+ flies (Fig. 1G), whose phenotype becomes only similar to that of sev-Poxn/+ flies when it is enhanced by a second copy of UAS-Poxn or by a heterozygous spa^{pol} background (Fig. 1H). By contrast, the phenotype of sev-Gal4/UAS-Poxn flies is similar to the sev-Poxn phenotype (Fig. 1A), whereas homozygous spa-Poxn flies appear wild type. Finally, in a heterozygous spa^{pol} background, spa-Poxn flies exhibit a weak phenotype similar to that of spa-Gal4/+; UAS-Poxn/+ flies (Fig. 1G), while in a homozygous spa^{pol} background, their phenotype is enhanced and similar to that of sev-Poxn/+; spa^{pol} flies (Fig. 1D), although clearly weaker (not shown).

**Ectopic expression of Pax proteins in eye-antennal discs interferes with Eyeless functions and produces headless flies**

To investigate the effect of ectopic transcription factors on early eye development, Pax proteins were expressed under the control of the eye-specific enhancer of the eyeless (ey) gene, a Pax6 homolog active in eye-antennal disc precursor cells (Quiring et al., 1994; Hauck et al., 1999). Thus, D-Pax2 was ectopically expressed in the developing eye disc under the indirect control of the eye-specific enhancer of ey, by the use of ey-Gal4 and UAS-D-Pax2 transgenes. As expected, this resulted in a dramatic interference with eye development, and no flies eclosed. Surprisingly, however, when the ey-Gal4/+; UAS-D-Pax2/+ pharate adults were examined, they not only lacked eyes, like the strongest known ey mutants (Quiring et al., 1994; Halder et al., 1998), but frequently had no head except for the proboscis (Fig. 2A), while thorax and abdomen were wild type (not shown). Similar phenotypes were observed when Poxn, Pox meso (Poxm), Gooseberry (Gsb) or Paired (Prd), i.e. Pax proteins whose paired domains belong to a class different from that of Ey or Pax6 (Noll, 1993), were ectopically expressed under the control of ey-Gal4 (Fig. 2A-D; and not shown). Flies transgenic for only the ey-Gal4 driver displayed a wild-type phenotype (not shown).

The headless phenotype is fully penetrant but exhibits variable expressivity. The phenotypes can be divided into four classes of decreasing strength: class I (5-15% of pharates) consisted of headless pharate adults that lacked all head structures derived from the eye-antennal discs (Fig. 2A); class II (25-60%) consisted of eyeless flies with most head structures and antennae absent (Fig. 2B); class III (40-65%) consisted of eyeless individuals with large parts of the head missing but one or both antennae present (Fig. 2C); while class IV consisted of flies with rough eyes of reduced but highly variable size (Fig. 2D) many of which eclosed spontaneously. Class IV phenotypes resemble hypomorphic ey mutants (Halder et al., 1998) and were found among ey-Gal4/+; UAS-Prd (UAS-Gsb, or UAS-D-Pax2)/+ flies (approx. 5-10%); when compared with approx. 1% of ey-Gal4/+; UAS-Poxn or UAS-Poxn/+ flies raised at 22°C at the expense of a reduced proportion of class I phenotypes. Although class I-III phenotypes do not eclose spontaneously – with the extremely rare exception of class III phenotypes – and die as pharate adults, they may live, even as class I headless phenotypes, for up to 2 days, if liberated at the right time from their pupal case.
A different small-eye phenotype is produced in UAS-Ey/+ flies, which depends on DNA-binding activities different from that of Ey. Left eyes of flies are shown in scanning electron micrographs. Headless flies result from interference with Ey functions, most of which eclose spontaneously. This result may imply that Ey becomes ectopic because of the perdurance of Gal4, and hence interferes with eye development at later stages.

These results suggest that Pax proteins that do not belong to the Ey class are able to interfere with functions of ey in the eye-antennal disc to generate headless flies. If true, it might be possible to rescue the headless phenotype by elevating the levels of the Ey protein. Indeed, one copy of UAS-Ey is able to rescue the headless phenotype of ey-Gal4/+; UAS-Ey/+ flies partially to produce small-eyed flies (Fig. 3A), characteristic for hypomorphic ey alleles (Halder et al., 1998). Although only about a quarter of the rescued flies eclosed, almost all pharate adults exhibit a small-eye phenotype and only few (<5%) a more severe class III phenotype. Interestingly, as previously observed in vertebrates (Schedl et al., 1996), the additional dose of UAS-Ey in the absence of ectopic Pax protein expression also results in flies with reduced eye size (Fig. 3B; Curtiss and Mlodzik, 2000; Plaza et al., 2001). However, this small-eye phenotype seems to be different from the class IV phenotype obtained after misexpression of Pax proteins because no headless flies or flies that lack eyes or other head structures are observed among the ey-Gal4/+; UAS-Ey/+ adults.

We conclude that the headless phenotype results from an interference with ey functions during development of the eye-antennal disc. Hence, we anticipated that complete absence of these functions might generate headless flies, i.e. a much more severe phenotype than that of previously analyzed ey alleles (Halder et al., 1998). This prediction has been confirmed by our analysis of strong ey mutants (Fig. 2E). As ey is activated by the product of its paralog twin of eyeless (toy) (Halder et al., 1998; Czerny et al., 1999), we tried to rescue the headless phenotype of ey-Gal4/UAS-Gsb flies by a UAS-Toy instead of a UAS-Ey transgene. However, these experiments showed no rescue, which suggests that the activity of the ey gene is close to its maximum level and hence higher Toy levels are unable to raise the concentration of Ey sufficiently.

**Early interference with ey functions is crucial for the generation of headless flies**

As expected from an analysis of the eye-specific enhancer of ey (Hauck et al., 1999), expression of ey-Gal4 is specifically expressed in the developing eye-antennal disc of the embryo and larva (not shown). This does not imply, however, that ectopic Pax proteins are able to interfere with ey functions during the entire development of the eye-antennal disc. To determine the period that is critical for producing a headless phenotype, we used the flip-out technique. D-Pax2 expression under the control of ey-Gal4 was induced by a heat shock activating Flipase (Flp) in the eye-antennal discs of y w hsp70-flp/+; ey-Gal4/+; UAS>y w >D-Pax2/+ embryos or larvae at different times of development. As evident from Table 1, the severity of the headless phenotypes is reduced with progressing time of initial D-Pax2 activation. Class I headless flies are generated only if D-Pax2 is induced before 12 hours AEL (after egg laying), i.e. at the time of ey activation in eye-antennal discs during early stage 15 (Hauck et al., 1999). The headless phenotypes clearly resulted from the heat-induced activation of ey-Gal4.

<table>
<thead>
<tr>
<th>Time*</th>
<th>4-12 hours</th>
<th>12-16 hours</th>
<th>16-24 hours</th>
<th>24-48 hours</th>
<th>48-96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharate adults‡</td>
<td>1-III</td>
<td>II,III</td>
<td>III</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Fraction of survivors</td>
<td>0.05</td>
<td>0.25</td>
<td>0.7</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Eclosed flies§</td>
<td>IV (0.6)</td>
<td>IV (0.18)</td>
<td>IV (0.08)</td>
<td>Rough clones (0.11)</td>
<td>No rough clones, Mosaic eyes</td>
</tr>
<tr>
<td>Wild type (0.4)</td>
<td>Wild type (0.82)</td>
<td>Wild type (0.92)</td>
<td>Wild type (0.89)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Time interval after egg laying during which D-Pax2 was heritably activated through the activation of flp recombinase under the control of the hsp70 promoter by a 30 minute heat shock at 37°C (Struhl and Basler, 1993) in eye-antennal discs of y w hsp70-flp/+; ey-Gal4/+; UAS>y w >D-Pax2/+ embryos or larvae.

‡Classes of headless phenotypes observed (compare with Fig. 2A-E).

§Fractions of spontaneously eclosed flies that exhibited a class IV small-eye phenotype, were wild type or had clones in the eye are indicated in parentheses.
the $D$-Pax2 transgene through Flp because all class IV phenotypes had lost the mini-white gene of the flip-out transgene, while all wild-type flies had retained it, as evident from their different eye colors. Class II phenotypes are observed only if $D$-Pax2 is activated before 16 hours AEL or the beginning of stage 17, while class III phenotypes are generated only if $D$-Pax2 activation in eye-antennal discs occurs before the end of embryogenesis (Table 1).

Induction of $D$-Pax2 expression in eye-antennal discs after embryogenesis produces no headless flies, which is surprising because this is the period of disc proliferation. Most flies obtained after $D$-Pax2 induction during the first larval instar are wild type, while few exhibit one or rarely more rough clones (induced 24-48 hours AEL in Table 1), which suggests that most clones expressing $D$-Pax2 in the eye disc are lost and compensated by proliferating wild-type cells. Therefore, induction of $D$-Pax2 in first instar eye-antennal discs still strongly inhibits cell proliferation. Later induction of $D$-Pax2, during the second or early third instar, produces adults with mosaic eyes whose mutant clones are not rough, which indicates that clones are no longer lost and develop normally.

We conclude that the critical period for producing a strong headless phenotype by interference with $ey$ functions occurs at the very beginning of $ey$ expression in the primordial eye-antennal disc, long before cell proliferation begins in first instar larvae (about 15-15 hours after hatching; Madhavan and Schneiderman, 1977). In addition, the strongest headless phenotypes result from a complete loss of all derivatives of the eye-antennal discs.

**How do ectopic Pax proteins interfere with $ey$ functions?**

Several models illustrating the mechanisms by which Pax proteins might interfere with $ey$ functions are conceivable (Fig. 4). In the simplest case, Pax proteins switch off $ey$ transcription, either directly (Fig. 4, model I) or indirectly. Alternatively, Pax proteins might act through a dominant negative mechanism (Herskowitz, 1987), either by binding to $ey$ or its partners (Fig. 4, model II) or by binding to $ey$ DNA-binding sites (Fig. 4, model III), thus preventing proper regulation of $ey$ target genes. Finally, Pax proteins might interfere with $ey$ functions through an entirely different mechanism by activating a genetic program that inhibits or counteracts the developmental pathway initiated by the network of $ey$ and its target genes (Fig. 4, model IV). In contrast to the interference by a dominant negative mechanism, this last mechanism of a ‘developmental pathway interference’ does not necessarily result in the misregulation of $ey$ target genes (Fig. 4).

The first model was ruled out because transcription of $ey$ remains unaffected in eye-antennal discs of $ey$-Gal4/+; UAS-$Gsb$/+ embryos (not shown). As the known target genes of $Ey$, $eyes absent$ ($eya$), $sine oculis$ ($so$) and $dachshund$ ($dac$), are activated in eye-antennal discs only during larval stages (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Shen and Mardon, 1997; Halder et al., 1998; Niimi et al., 1999; Kumar and Moses, 2001), we could not test whether the state of $ey$ target genes during the critical period is consistent with dominant negative mechanisms (Fig. 4, models I and III). However, the fact that eye and head development are normal when ectopic expression of Pax proteins occurs after embryogenesis (Table 1) argues that these ectopic proteins are unable to inhibit transcription of $eya$, $so$ and $dac$ because their function becomes crucial for eye development only during the

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**Fig. 4.** Mechanisms of interference with $Ey$ functions through ectopic Pax proteins. Four mechanisms by which ectopic Pax proteins could interfere with the developmental program depending on $Ey$ functions in eye-antennal discs are illustrated. In the first model, Pax proteins repress $ey$ transcription either directly by blocking its enhancer (I), or indirectly by interfering with genes or their products required for $ey$ activation (not shown). In the second (II) and third model (III), Pax proteins inhibit transcription of $Ey$ target genes ($X$), activated in the wild type (wt) by $Ey$ and a set of transcription factors (C), in a dominant negative manner. By contrast, in the fourth model (IV), ectopic Pax proteins do not interfere with transcription of $ey$ or that of the targets of its product. Rather by altering the regulation of a set of target genes ($Y$), in combination with a set of transcription factors (D), ectopic Pax proteins activate a genetic program that interferes with the normal progression of the developmental pathway dependent on $ey$. While our results exclude models I-III and favor model IV of ‘developmental pathway interference’ activated by ectopic Pax proteins or other ectopic transcription factors, they do not rule out models I-III for few specific transcription factors not examined in this study.
Generation of headless phenotype by Gsb depends on functional paired and transactivation domains

To this end, several transgenes encoding mutated Gsb proteins were expressed under the control of ey-Gal4, and their ability to generate a headless phenotype was recorded (Fig. 5). If the paired domain or the entire C-terminal moiety of Gsb, which includes transactivation but no DNA-binding domains (Xue et al., 2001), is deleted, only wild-type animals are produced, whereas the mere removal of the homeodomain generates class III and IV headless phenotypes. Accordingly, the ability of Gsb to interfere with Ey functions in the eye-antennal disc completely depends on its DNA-binding paired domain and its transactivation domains. However, there is no strict requirement for the homeodomain, although its presence enhances the interference of Gsb with Ey functions.

Although these results favor model IV, which depends on both the DNA-binding and activation ability of Pax proteins, they do neither exclude III or II (Fig. 4). For Gsb might be able to recognize some Ey binding sites even in the absence of its homeodomain, and binding of Gsb to Ey might depend on both its paired domain and C-terminal portion. Therefore, a point mutation was introduced into the paired domain of Gsb known to abolish its DNA-binding ability in vitro (Xue et al., 2001). This mutated GsbP17L protein is unable to interfere with Ey functions in the developing eye-antennal disc (Fig. 5), which suggests that DNA binding of Gsb through its paired domain is crucial to produce a headless phenotype and consequently renders a mechanism by which Gsb interferes with Ey function by binding to Ey protein very improbable (Fig. 4, model II). By contrast, swapping the C-terminal moiety or the N-terminal paired domain and homeodomain of Gsb with the corresponding portions of Poxn has no effect and produces the same spectrum of headless phenotypes as wild-type Gsb protein (Fig. 5). Thus, neither the origin of the C-terminal transactivation domain nor the DNA-binding specificity of the paired domain appear to be crucial, although both are required, to produce headless flies. This conclusion is consistent with our observation that ectopic expression of Pax proteins whose paired domains differ in DNA-binding specificity from that of Ey and Toy are equally effective in producing a headless phenotype, and argues against a dominant negative mechanism in which Pax proteins compete for Ey DNA-binding sites (Fig. 4, model III), but does not exclude it rigorously.

Transgene | Protein | Phenotype
--- | --- | ---
UAS-Gsb | P | I - IV
UAS-GsbΔP | | wt
UAS-GsbΔH | | III - IV
UAS-GsbN | | wt
UAS-GsbC | | wt
UAS-GsbP17L | L | wt
UAS-GsbN+PoxnC | | I - IV
UAS-PoxnN+GsbC | | I - IV

Fig. 5. Generation of headless phenotype depends on functional paired domain and transactivation domain in ectopic Gsb. The ability of mutated Gsb proteins, which are encoded by the transgenes listed in the left column and whose structure is shown schematically in the middle column, to generate class I-IV headless phenotypes is indicated in the right column. For a detailed explanation, see text.

Headless flies are produced by developmental pathway interference

The dominant-negative mechanism implies that the ectopic Pax proteins giving rise to a headless phenotype compete with Ey for the same DNA-binding sites (Fig. 4, model III). Such a mechanism seems improbable because the DNA-binding specificities of Ey and Pax proteins that do not belong to the Ey/Pax6 class differ considerably (Czerny and Busslinger, 1995). However, model III (Fig. 4) could be ruled out more strictly if changing the DNA-binding specificity of the ectopically expressed Pax protein to that of Ey produced a weaker phenotype than that observed after ectopic expression of the wild-type Pax protein. Therefore, by taking advantage of the observation that the DNA-binding specificity of Pax proteins depends only on three amino acids at positions 42, 44 and 47 of the paired domain (Czerny and Busslinger, 1995), the DNA-binding specificity of Gsb was converted to that of Ey by mutating these three amino acids Q, R and H of Gsb to I, Q and N, which are specific for Ey. None of six independent UAS-GE lines that expressed this mutated Gsb protein under the control of ey-Gal4 showed a headless phenotype. Although four lines displayed a phenotype (Fig. 3C) very similar to wild type (Fig. 3D), the remaining two lines exhibited a weak small-eye phenotype (line 5; not shown) similar to that of ey-GAL4/+; UAS-Ey/+ flies (Fig. 3B) or a class III-IV phenotype (line 4; not shown), which indicates that the activity of the mutated Gsb protein was altered to that of Ey or to a slightly dominant negative form of Ey, presumably caused by its Gsb transactivation domains (Xue et al., 2001). These results argue strongly against a dominant negative mechanism by which Pax proteins different from Ey and Toy interfere with ey functions in the early eye-antennal disc (Fig. 4, models II and III). Hence, interference with the normal developmental pathway (Fig. 4, model IV) is indeed the only mechanism that is able to explain the generation of headless flies.
Ectopic expression of many transcription factors is able to generate headless flies

Our conclusion that Pax proteins, ectopically expressed under the control of ey-Gal4, generate headless flies by activating a genetic program that interferes with that of the eye-antennal disc raises the possibility that this property is not specific for Pax proteins, but is shared by many transcription factors. Therefore, we tested if other transcription factors had a similar effect on head development when they were expressed under ey-Gal4 control. As evident from Table 2, all transcription factors that were tested had at least a small effect. Indeed, the MADS domain protein MeF2, which is important for myoblast fusion and muscle differentiation (Lin et al., 1997), is even more potent than Pax proteins in producing the headless phenotype; the bHLH transcription factor Sim, which specifies development of the ventral midline cells in the embryo (Nambu et al., 1990), is equally effective. By contrast, another bHLH protein, Ato, a proneural gene product required for development of chordotonal organs and photoreceptors (Jarman et al., 1995), hardly interferes with head development and only slightly reduces eye size. In view of the fact that Ato is expressed in all cells anterior to the morphogenetic furrow and in the proneural cluster from which the photoreceptor R8 is selected, its inability to interfere with the genetic program initiated by Ey in the eye-antennal disc may not be surprising. However, another transcription factor important during the development of eye discs and the product of a gene that may be a direct target of Ey, Dac (Shen and Mardon, 1997), strongly interferes with head development when it is expressed prematurely in eye discs of embryos, rather than at its normal time during the third instar. Two additional transcription factors that were ectopically expressed under ey-Gal4 control, the homeodomain proteins En and Ubx, were unable to produce headless flies, but interfered, though at low penetrance, with eye development to produce small-eyed flies (Table 2).

These results show that ectopic expression of many transcription factors interferes with the developmental program of eye-antennal discs and supports our hypothesis of a developmental pathway interference mechanism.

Developmental pathway interference inhibits cell proliferation that may be overcome by Cyclin E

The eye-antennal discs of ey-GAL4/+; UAS-Gsh/+ third instar larvae are absent or strongly reduced in size (not shown). Evidently, developmental pathway interference induced by the ectopic expression of transcription factors eventually results in the inhibition of cell proliferation and/or apoptosis in these discs. To investigate which of these two processes is responsible for the generation of headless flies, we tried to inhibit apoptosis or to stimulate cell proliferation in eye-antennal discs. While inhibition of apoptosis by the expression of the baculovirus P35 protein (Hay et al., 1994) is unable to suppress the headless phenotype (not shown), stimulation of cell proliferation by the expression of D-Myc suppresses it in spontaneously eclosing adults (5-20%), producing adults of variable eye size, from eyeless adults (Fig. 6A) to adults whose eye size is only slightly reduced (Fig. 6B). The headless
The document discusses the role of ectopic transcription factors in developmental pathway interference. It highlights how the expression of these factors can altering cell proliferation and influence cell differentiation, particularly in the context of eye development in Drosophila. The text mentions the use of genetic tools such as transgenic flies with targeted expression of transcription factors to study these effects. The results show that ectopic expression of transcription factors can inhibit cell proliferation and lead to specific phenotypes, such as headless flies or flies with small eyes. The document also points out the implications of these findings for understanding evolutionary pathways and the role of developmental mechanisms in both normal development and evolutionary change.
Developmental pathway interference results from the inhibition of a developmental program

Recently, it has been shown that ectopic expression of Antp in the eye disc inhibits eye development and generates eyeless flies (Plaza et al., 2001). On the basis of in vitro binding studies, it has been proposed that Antp as well as other homeodomain proteins exert this effect by binding through their homeodomain to the paired domain and homeodomain of the Ey protein, thus inhibiting the activation of Ey target genes in a dominant negative manner, as illustrated in model II of Fig. 4. Several of our results strongly suggest that the mechanism inhibiting eye and head development by the ectopic expression of a transcription factor does not crucially depend on the dominant negative interaction of an ectopic homeodomain factor with the Ey protein, but is of a more general nature. (1) When tested in vivo for its ability to generate headless or eyeless flies, the Gsb protein strictly depends on its paired domain without which it does not affect eye development, while in the absence of its homeodomain it is still able to produce eyeless flies. (2) A truncated Gsb protein, which consists of both DNA-binding domains, the paired domain and the homeodomain, but lacks its transactivation domains, has no effect on eye or head development. (3) If a missense mutation is introduced that abolishes the DNA-binding activity of its paired domain but does not affect its homeodomain, Gsb is unable to interfere with eye development. (4) Similarly, if the DNA-binding specificity of the paired domain of the ectopic Gsb is altered to that of Ey, its interference with head and eye development is abolished or reduced to that of ectopic Ey. (5) The two homeodomain proteins tested that have no paired domain, Ubx and En, inhibit eye development relatively weakly and with low penetrance. In fact, they exhibit the weakest phenotype (class IV) of all transcription factors examined (Table 2). (6) Many non-homeodomain transcription factors inhibit eye and head development very efficiently. (7) While elevating Ey levels may overcome the inhibition of some ectopic transcription factors, this is not the case for Sim (not shown) and perhaps for several of the other factors tested. (8) By contrast, the inhibition of eye and head development by ectopic transcription factors can be reduced or entirely removed by elevating the concentrations of CycE or Myc. (9) Interference with eye and head development is limited to a critical short period in the embryonal eye-antennal primordium, long before the Ey targets so,eya and dac are activated in the larva (Kumar and Moses, 2001).

Taken together, our results demonstrate that the observed inhibition of eye and head development by an ectopic transcription factor cannot be explained by its interaction with Ey protein, but rather is caused by a block in the execution of the developmental program primed by Toy and Ey (Czerny et al., 1999; Gehring and Ikeo, 1999). In addition, they raise the possibility that the decisive inhibition by Antp does not occur through its binding to Ey (Plaza et al., 2001), but through this mechanism of developmental pathway interference.
Early interference with the Ey pathway generates headless flies

It is important to note that complete interference with eye-antennal development primed by Toy and Ey produces headless flies that lack all structures derived from the eye-antennal disc, a phenotype that is much stronger than that reported for ey loss-of-function alleles (Halder et al., 1998). We have shown that its primary cause is a block during the G1 phase of the cell cycle, because in some cases it can be completely removed by overexpression of CycE. This block can occur only at a very early stage of eye-antennal disc development, which suggests that Toy and Ey prime eye-antennal development in the corresponding embryonic disc primordium, long before the fates of eye and antenna are specified during the second instar (Kumar and Moses, 2001). In agreement with such an early role for Ey in the development of both eye and antenna, Ey is expressed throughout the eye-antennal disc of the embryo and first instar larva (Quiring et al., 1994; Kumar and Moses, 2001). If Toy and Ey prime the genetic program that activates the network regulating development not only of the eye (Czerny et al., 1999), but also of the antenna, one would expect that ey mutants that lack any function in the eye-antennal disc would also display a headless phenotype. Indeed, strong ey mutants show a phenotype indistinguishable from the headless phenotype produced by interference with eye-antennal mutants show a phenotype indistinguishable from the headless phenotype encoded by pair-rule genes. A classical example is the blastoderm stage are those between transcription factors early developmental pathway boundaries established at the developmental program of the adjacent domain. Examples of necessity of the factors to avoid interference with the abundant during development and may result from the their domains of expression. Indeed, such boundaries are observed in mouse embryos in which ey-Gal4 gives rise to eyeless adults. We have examples of sharp boundaries are observed in mouse embryos between different types of paired domain transcription factors, such as between Pax2 and Pax6 (Goulding et al., 1993) and between Pax3 and Pax6 (Schwarz et al., 1996) in the developing neural tube.

Do successful alterations of the genetic program require multiple changes?

Ectopic expression of a transcription factor during development, as shown here, usually provides no selective advantage to the organism, but is deleterious. Clearly, expression of a single transcription factor in a new spatiotemporal pattern is probably only very rarely successful during evolution. Activation of more than one transcription factor at the same time and location might be more probable to circumvent developmental pathway interference. An exciting mechanism through which this might be achieved is the simultaneous activation of several signal transduction pathways (Rutherford and Lindquist, 1998). Here, cell fates are altered without the induction of a block in the cell cycle and apoptosis, similar to the situation in rare dominant homeotic mutants or in cases in which ectopic expression of a single transcription factor suffices to alter the developmental pathway into one that exists elsewhere. The ectopic factor might avoid interference by repressing the endogenous program while activating its own.

Are cell cycle checkpoints linked to developmental and evolutionary checkpoints?

Cells are monitored continuously during development for improper specification of cell fate and may respond to incompatible combinations of active signaling pathways and transcription factors by the induction of apoptosis (Bonini and Fortini, 1999). Consistent with this view, our results suggest that apoptosis is induced when ectopic transcription factors interfere with differentiation pathways. However, we find that if interference occurs before or during the cell proliferation stage of a developmental pathway, it induces a block in the cell cycle rather than apoptosis, because overexpression of CycE, but not of the P35 inhibitor protein of apoptosis, can override it. As a consequence of this block, cells may eventually induce apoptosis.

It appears that in many instances, interference at the CycE-sensitive checkpoint of the cell cycle is more efficient or occurs at additional checkpoints of the cell cycle (Hartwell, 1991), because overexpression of Ey or CycE only partially overrides the block induced by some ectopic transcription factors, and overexpression of Myc can be more efficient than that of CycE in by-passing the block (Table 2). Thus, the quality control mechanism may occur during various checkpoints of the cell cycle and induce a block in the cell cycle during the proliferation stage followed by apoptosis, or directly induce apoptosis during the differentiation stage. Hence, linking control of developmental pathways to cell cycle checkpoints extends the checkpoint concept to development as well as evolution.

Note added in proof

Plaza et al. (2001) reported that expression of UAS-Antp under the control of ey-Gal4 gives rise to eyeless adults. We have
repeated these experiments by crossing ey-Gal4 virgins with UAS-Antp males and found that no adult flies eclosed (<1%). All ey-Gal4/UAS-Antp flies die and are present in about equal portions as headless (classes I and II) and eyeless (class III) pharate adults. Overexpression of D-CycE rescues these to adults that eclosed spontaneously (about 10%) or to pharate whose phenotype is weakened on average by at least two phenotypic classes (+/+; compare with Table 2). These results demonstrate that early ectopic expression of Antp in eye-antennal discs inhibits also both eye and head development, and prove the correctness of our conjecture that the crucial inhibition by Antp does not occur through its binding to Ey, as has been suggested (Plaza et al., 2001), but rather as a consequence of pathway interference in agreement with the results shown here.

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