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Headless flies generated by developmental pathway interference

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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*

INTRODUCTION

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 and may induce apoptosis. We conclude that expression of most transcription factors in a new spatiotemporal pattern interferes with the established developmental pathway and propose the existence of a surveillance mechanism that selects against most changes in the genetic program during evolution.

MATERIALS AND METHODS

Construction of transgenes

sev-D-Pax2 was prepared by cloning the 2.8 kb *Eco*RI-*Xba*I fragment of the *D-Pax2*-cDNA cpx1 (Fu and Noll, 1997), including the 5′-leader and entire coding region, into a vector derived from pUAST, in which the $(UAS)_5$ enhancer and *hsp70* minimal promoter (Brand and Perrimon, 1993) had been replaced by a 2x*sev* enhancer-*sev* promoter

cassette (from E. Hafen). *spa-Gal4* was prepared by cloning the 7.1 kb *Eco*RI genomic fragment of *D-Pax2* (Fu and Noll, 1997), extending from intron 2 into intron 4 and including the *spa* enhancer, into the *Not*I site of the pDA188.1 vector (a P element vector including the *hsp70* minimal promoter, the Gal4-coding region and the *tubulinα1* trailer, prepared and provided by D. Nellen and K. Basler). To construct *spa-Poxn*, a 1.73 kb fragment, extending from an *Eco*RI site introduced 23 bp upstream of the coding region of the P4c6 *poxn*-cDNA to an artificial *Eco*RI 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 *D-Pax2* in bluescript SK⁻. After inserting upstream of the promoter the 926 bp *Spe*I fragment including the *spa* enhancer (Fu and Noll, 1997), the complete insert was transferred as 3.26 kb *Xba*I-*Kpn*I fragment into the P-element vector pW6 (Klemenz et al., 1987) to produce *spa-Poxn*.

UAS-Poxn was prepared by combining the *Eco*RI-*Bst*XI 5′-fragment from pSK⁻-PoxnPD (an *Eco*RI subclone encoding the paired domain of Poxn, obtained by PCR amplification of the P4c6 *poxn*-cDNA with the primers 5′-CATCGAATTCATGCCGCACACAGGT-CAA-3′ and 5′-GCGGAATTCTACTGACTGGATGTCATCTC-3′, in the bluescript vector pSK⁻) with the *Bst*XI-*Eco*RI 3′-fragment from the P4c6 *poxn*-cDNA clone (Dambly-Chaudière et al., 1992) into the *Eco*RI site of pUAST (Brand and Perrimon, 1993). *UAS-Gsb*, *UAS-Prd* and *UAS-Poxm* were obtained by cloning the *gsb*-cDNA BSH9c2 (Baumgartner et al., 1987), the *prd*-cDNA c7340.6 (Frigerio et al., 1986) and the 2.6 kb *poxm*-cDNA P29c1 (Bopp et al., 1989), respectively, into the *Eco*RI site of pUAST. *UAS-D-Pax2* was constructed by cloning the 2.8 kb *Eco*RI-*Xba*I 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 *Eco*RI insert of a nearly full-length *toy*-cDNA clone (10.4) isolated from an eye disc cDNA library in λgt10 (prepared by A. Kowman and provided by G. M. Rubin).

UAS>w⁺>D-Pax2 was constructed by first cloning the 2.8 kb *Eco*RI-*Xba*I fragment of the *D-Pax2*-cDNA cpx1 into the *Eco*RV site of pKB342 (a bluescript vector including the trailer of *tubulinα1*; provided by K. Basler). The insert, which consisted of the *D-Pax2*-cDNA and the *tubulinα1* trailer, was removed as a *Kpn*I-*Xba*I fragment and cloned into pM51, a Carnegie 2-derived P-element vector (Rubin and Spradling, 1983) analogous 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 *Kpn*I fragment from pKB340 (pUC19 clone of direct FRT repeats flanking the *hsp70* trailer and mini-white gene; provided by K. Basler), into the *Kpn*I 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 by the use of two sets of primers: gsbprdbox5′ (5′-ACCGGAATTCATGGCTGTTTCG-GCTCTC-3′) with GE2 (5′-GAGACGCAGCCATTAGAGACCTGC-AGGATGCGGG-3′) and GE1 (5′-CCCGCATCCTGCAGGTCTCT-AATGGCTGCGTCTC-3′) with gsbprdbox3′ (5′-CCGGTCGA-CTAGCCGTCGATGCTGTGGGA-3′). *gsb* cDNA BSH9c2 was used as template. Subsequently, 1/50 of the volume of each PCR reaction were combined, amplified by PCR with the gsbprdbox5′ and gsbprdbox3′ primers, and inserted as *Eco*RI-*Sal*I fragment into bluescript pKS⁻ for sequence verification. Finally, the 0.43 kb *Eco*RI-*Bam*HI fragment of this insert was combined with the 1.05 kb *Bam*HI-*Eco*RI from BSH9c2 in the *Eco*RI 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-GsbN*, *UAS-GsbC* and *UAS-GsbN+PoxnC* were prepared by removing the inserts from the corresponding pAR clones (Xue and Noll, 1996; Xue et al., 2001) by *Xba*I and *Nhe*I digestion and ligation in the proper orientation into the *Xba*I 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 *Eco*RI insert of pKSpL5-GsbP17L (Xue et al., 2001) into pGEM-2 in such an orientation that it could be removed as *Xba*I-*Nhe*I fragment for ligation into pUAST. Finally, to obtain *UAS-PoxnN+GsbC*, its *Eco*RI 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 *Eco*RI-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′-AATTAACCCTCACTAAAGGG-3′ (T3 primer) and 5′-GACCGCT-GTTGCGCAGAATC-3′ (poxnP-7 primer), and subsequent ligation with the 750 bp *Fsp*I-*Eco*RI fragment of the BSH9c2 *gsb*-cDNA (Baumgartner et al., 1987), which encodes the C-terminal moiety of Gsb, into the *Eco*RI 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-Poxn/TM3*, *Sb* (Dambly-Chaudière et al., 1992),
w; *sev-Poxn/TM3*, *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*I fragment of intron 4 of *D-Pax2*; Fu and Noll, 1997),
w; *spa-Poxn*,
w; *spa-Poxn*; *spa^pol^*,
w; *sev-Gal4* (3rd chromosome; from E. Hafen),
w; *UAS-Poxn-6* (2nd chromosome),
w; *UAS-Poxn-5* (3rd chromosome),
y 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-Poxm* (3rd chromosome),
w; *UAS-Prd-1* (3rd chromosome),
w; *UAS-Toy-6/TM3*, *Sb*,
y w; *UAS-Ey/TM3*, *Sb* (Halder et al., 1995),
w; *UAS-Gsb-7/CyO*; *UAS-Ey/TM3*, *Sb*,
y w hsp70-flp,
w; *ey-Gal4*; *UAS>w⁺>D-Pax2-1*,
w; *UAS-GE-8* (3rd chromosome),
w; *UAS>w⁺>D-Pax2-1* (2nd 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-GsbC-1* (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* (2nd chromosome; Xiao et al., 1996),
w; *UAS-D-Myc* (3rd chromosome) (Johnston et al., 1999),

w; *UAS-D-CycE* (3rd chromosome; from C. Lehner),
w; *UAS-P35*; *UAS-Gsb-1*,
w; *UAS-Gsb-7*; *UAS-D-Myc*,
w; *UAS-Gsb-7/CyO*; *UAS-D-CycE*,
w; *ey-Gal4*; *UAS-D-Myc/TM3, Sb*,
w; *ey-Gal4*; *UAS-D-CycE*.

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} flies (Fig. 1C). An even more dramatic phenotype is produced when both *D-Pax2* genes are replaced by spa^{pol} alleles (Fig. 1D). This phenotype is much 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.

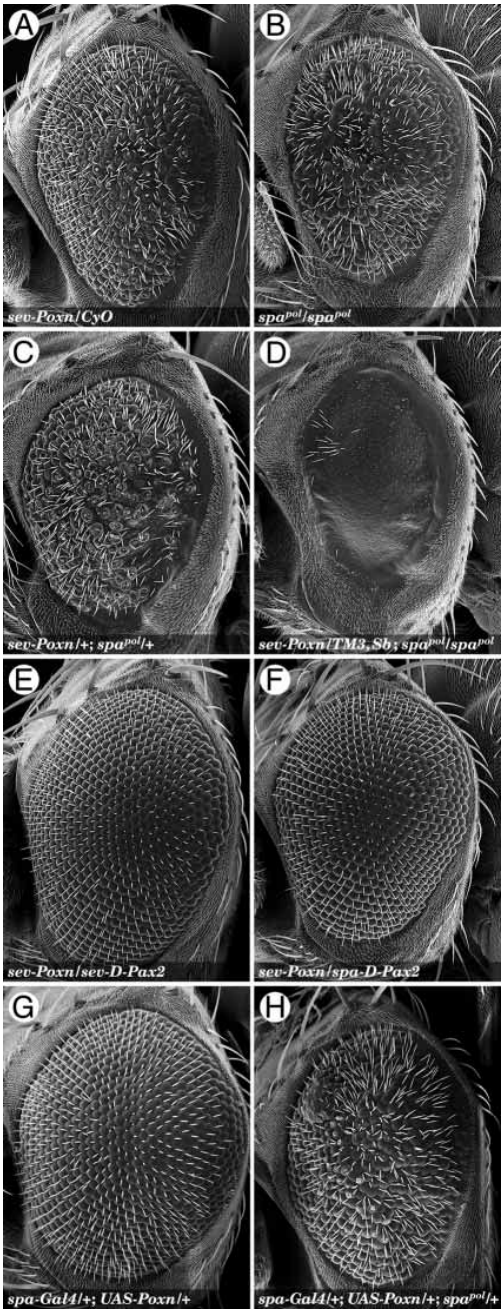

Fig. 1. Interference of ectopic Poxn with D-Pax2 functions in eye development. Left eyes of flies of the genotype indicated in each panel are shown in scanning electron micrographs. Note that the phenotype produced by ectopic Poxn expression under the control of the *sev* enhancer (A) is similar to that of spa^{pol} mutants (B), but stronger than that generated by ectopic Poxn expressed under the indirect control of the *spa* enhancer of *D-Pax2* (G). Reducing *D-Pax2* expression during eye development in heterozygous (C,H) or homozygous spa^{pol} (D) backgrounds enhances the phenotypes produced by ectopic Poxn, whereas raising D-Pax2 levels by an additional copy of *D-Pax2* under the control of the *sev* (E) or *spa* (F) enhancer rescues the *sev-Poxn* phenotype to wild type (compare with Fig. 3D).

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 *sev* 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).

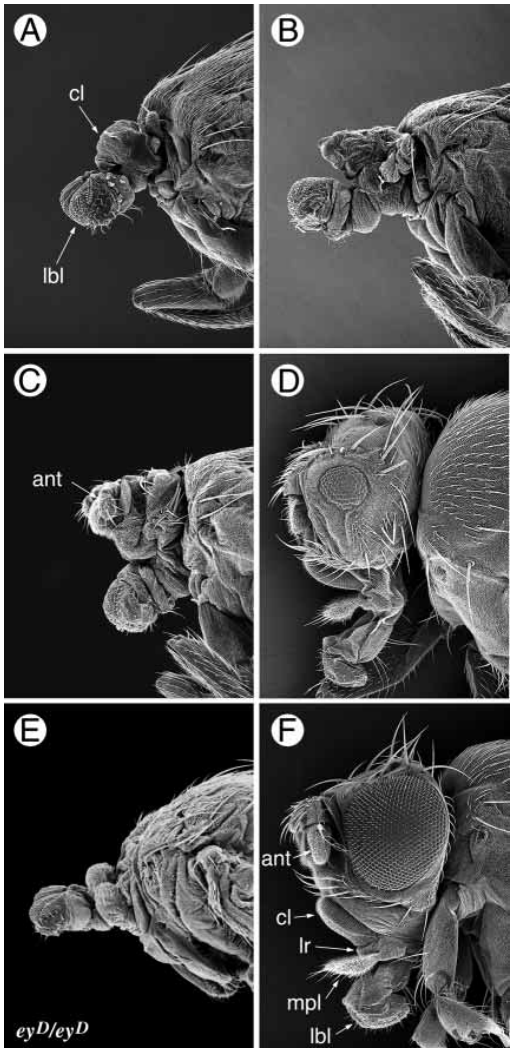

Fig. 2. Ectopic Pax proteins in eye-antennal disc generate headless flies. Scanning electron micrographs of the anterior portion of *ey-Gal4/+*; *UAS-Gsb-1/+* (A-D), homozygous ey^D (E) and wild-type flies (F) are compared. The interference with head development of ectopic Gsb (D-Pax2, Poxn, Poxm, or Prd) in eye-antennal discs results in 'headless' flies of variable expressivity. Representative phenotypes of the four phenotypic classes are shown: (A) class I, all head structures derived from eye-antennal disc, including eye, antenna, head capsule and maxillary palps, are missing, only the proboscis, largely derived from the clypeolabral (cl) and labial disc (lbl), is present; (B) class II, most head structures and both eyes and antennae absent; (C) class III, large parts of head and both eyes missing, portions of one or both antennae (ant) present; and (D) class IV, most of head and one or both eyes of reduced size present. Note that flies homozygous for the strong ey^D allele also exhibit a headless phenotype (E). ant, antenna; cl, clypeus; lbl, labellum; lr, labrum; mpl, maxillary palp.

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). Very 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 both 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-Poxm/+* 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.

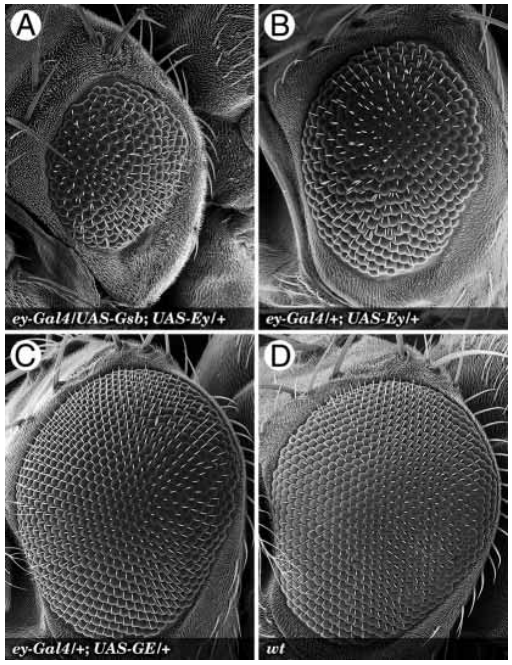

Fig. 3. Headless flies result from interference with *ey* functions, which depends on DNA-binding activities different from that of Ey. Left eyes of flies are shown in scanning electron micrographs. (A) *UAS-Ey* rescues the headless phenotype in *ey-Gal4/UAS-Gsb-7*; *UAS-Ey/+* flies almost completely to a small-eye phenotype. (B) A different small-eye phenotype is produced in *ey-Gal4/+*; *UAS-Ey/+* flies. (C) *ey-Gal4/+*; *UAS-GE-8/+* flies, which carry mutations in amino acids 42 (Q mutated to I), 44 (R to Q) and 47 (H to N) in the paired domain of *UAS-Gsb* changing its DNA-binding specificity to that of the Ey paired domain, exhibit little or no interference with *ey* functions and display, in four out of six lines, a phenotype similar to wild type (D) or, in two lines, a weak phenotype similar to *ey-Gal4/+*; *UAS-Ey/+* flies (B).

These results suggest that Pax proteins that do not belong to the Ey class are able to interfere with the 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-Gsb* 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 eclose, 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, most of which eclose spontaneously. This result may imply that the Ey concentration in eye-antennal discs is crucial for normal head development or that Ey becomes ectopic because of the perdurance of Gal4, and hence interferes with eye development at later stages.

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>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

Table 1. Critical period of interference with *ey* functions occurs during embryogenesis

	Time*				
	4-12 hours	12-16 hours	16-24 hours	24-48 hours	48-96 hours
Pharate adults‡	I-III	II,III	III	–	–
Fraction of survivors	0.05	0.25	0.7	1.0	1.0
Eclosed flies§	IV (0.6) Wild type (0.4)	IV (0.18) Wild type (0.82)	IV (0.08) Wild type (0.92)	Rough clones (0.11) Wild type (0.89)	No rough clones, Mosaic eyes

*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>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 13-15 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 II and III). However, the fact that eye and head development are normal when ectopic expression of Pax proteins occurs after

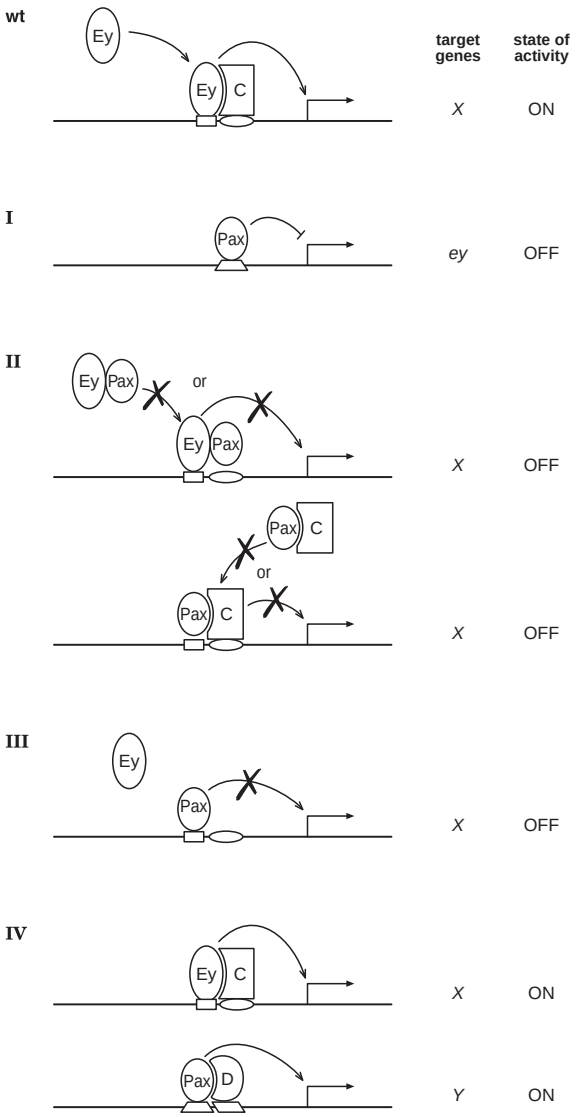

Fig. 4. Mechanisms of interference with Ey functions through ectopic Pax proteins. Four mechanisms by which ectopic Pax proteins could interfere with the developmental program depending on Ey functions in eye-antennal discs are illustrated. In the first model, Pax proteins repress *ey* transcription 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.

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

second instar (Kumar and Moses, 2001). Nevertheless, our observation that all ectopically expressed proteins that were tested and produced a headless phenotype included a paired domain, might suggest that these proteins interact with Ey through their paired domain in support of a dominant negative mechanism (Fig. 4, model II). A test of this hypothesis in the yeast two-hybrid system was negative and revealed no interactions between paired domains (not shown), a finding that is in agreement with an earlier demonstration that full-length Prd protein does not form homodimers, as assayed by the yeast two-hybrid system (Miskiewicz et al., 1996). As this test does not exclude the possibility that the paired domains of Pax proteins and Ey interact with each other in eye-antennal discs, we have tried to distinguish between models II-IV (Fig. 4) by altering the structure of an interfering Pax protein expressed in eye-antennal discs.

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.

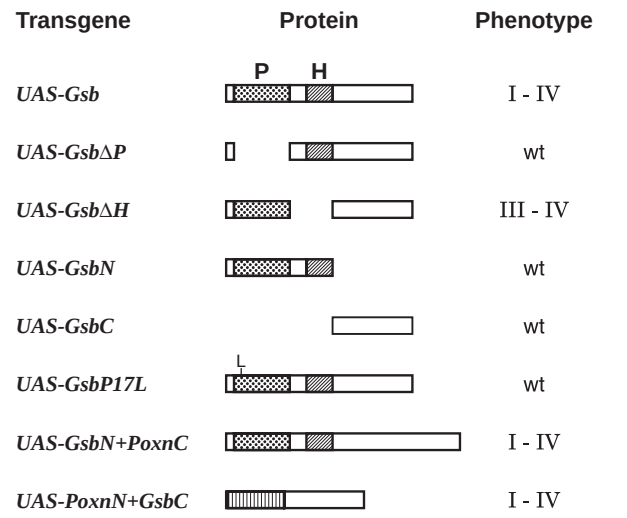

Fig. 5. Generation of headless phenotype depends on functional paired domain and transactivation domain in ectopic Gsb. The ability of mutated Gsb proteins, which are encoded by the transgenes listed in the left column and whose structure is shown schematically in the middle column, to generate class 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.

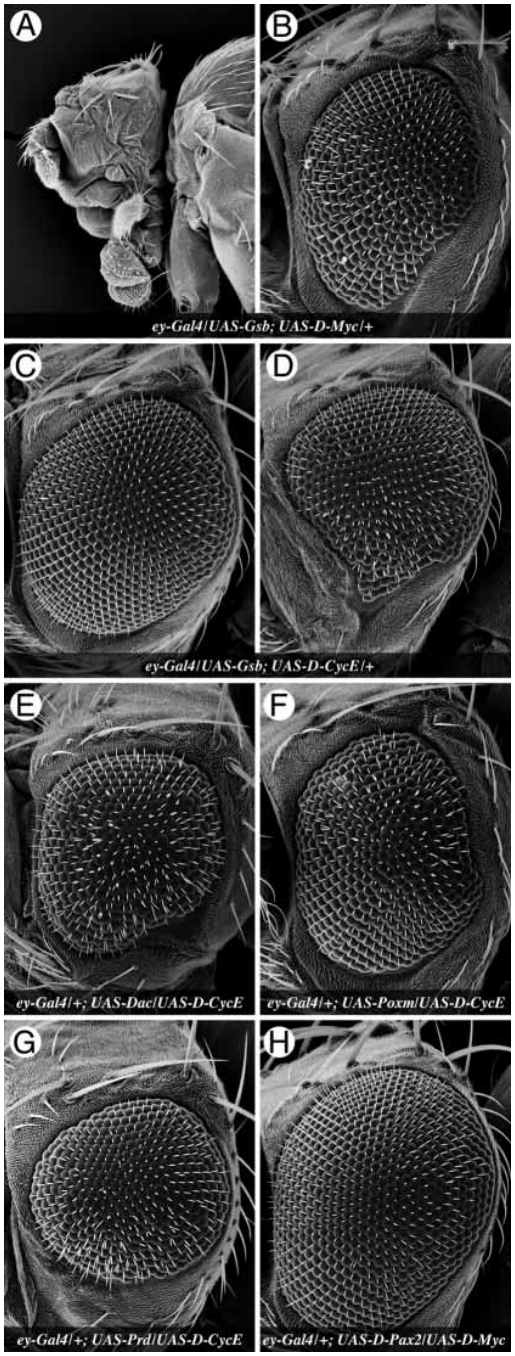

Fig. 6. Partial rescue by CycE or Myc of headless phenotype caused by developmental pathway interference. The rescue effect of D-Myc and D-CycE on adult head development, inhibited by ectopic expression of transcription factors under the control of *ey-Gal4*, is shown in scanning electron micrographs (anterior is to the left). (A,B) *UAS-D-Myc* rescues head (A) and eye development (B) in *ey-Gal4/UAS-Gsb-7*; *UAS-D-Myc/+* flies. (C,D) Complete (C) and partial (D) rescue of head and eye development by *UAS-D-CycE* in *ey-Gal4/UAS-Gsb-7*; *UAS-D-CycE/+* flies. (E-G) Partial (E) and nearly complete (F,G) rescue of head and eye development by *UAS-D-CycE* to adults that eclosed spontaneously in *ey-Gal4/+*; *UAS-Dac/UAS-D-CycE* (E), *ey-Gal4/+*; *UAS-Poxm/UAS-D-CycE* (F), and *ey-Gal4/+*; *UAS-Prd-1/UAS-D-CycE* flies (G). (H) Complete rescue of head and eye development by *UAS-D-Myc* in *ey-Gal4/+*; *UAS-D-Pax2-1/UAS-D-Myc* flies. Flies shown developed at room temperature (22°C), except for E, which developed at 25°C.

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-Gsb/+* 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

Table 2. Headless flies generated by ectopic transcription factors and their rescue by D-Myc or D-CycE

Transcription factor*	DNA-dinding domain‡	Phenotype§	Rescue efficiency¶ of	
			UAS-D-Myc	*UAS-D-CycE*
Dac	novel	I-IV**	–	+
Ubx	HD	IV‡, Wild type (0.94)	nd	nd
En	HD	IV‡, Wild type (0.84)	nd	nd
Ato	bHLH	IV‡	nd	nd
Mef2	MADS	I-II, (III)	++	+++
Sim	bHLH	I-III	+	+
D-Pax2	PD	I-IV	++++	+++
Poxm	PD	I-III	+	+++++
Poxn	PD	I-III	+	+
Prd	PD	I-IV	+	+++++
Gsb§§	PD	II-IV	+	++++

*Transcription factors expressed ectopically in eye-antennal discs of flies obtained by crossing *ey-Gal4* virgins with *UAS-Dac/TM3, Sb*, *UAS-Ubx/TM3, Sb Ser, lethal/TM6*, *UAS-En*, *UAS-Ato/CyO*, *UAS-Mef2*, *UAS-Sim*, *UAS-D-Pax2-1*, *UAS-Poxm*, *UAS-Poxn-6*, *UAS-Prd-1*, *UAS-Gsb-1* and *UAS-Gsb-7* males. For rescue of the headless phenotype with D-Myc or D-CycE, virgins were *ey-Gal4*; *UAS-D-Myc/TM3, Sb* or *ey-Gal4*; *UAS-D-CycE*.

‡DNA-binding domain of ectopically expressed transcription factor: HD, homeodomain; bHLH, basic helix-loop-helix domain; MADS, MADS domain; PD, paired domain.

§Classes of headless phenotypes observed after development at room temperature.

¶Rescue efficiencies by one copy of *UAS-D-CycE* or *UAS-D-Myc* are computed as increase in weighed averages of phenotypic classes observed, each + sign corresponding to an increase by half a class; nd, not determined.

**None of the adults eclosed. One copy of *UAS-D-Myc* deteriorated the phenotype by half a class, as indicated by a minus sign, while the presence of *UAS-D-CycE* produced larger eyes in the adults some of which eclosed spontaneously at 25°C (compare with Fig. 6E).

‡‡Adults exhibit a very weak class IV phenotype and eclose all spontaneously. Fractions of wild-type flies are indicated in parentheses.

§§Data shown for *UAS-Gsb-7* line. The stronger *UAS-Gsb-1* line (Fig. 2A-E) produces class I-IV phenotypes (with frequencies indicated in the text). Its rescue efficiencies at 25°C by D-Myc and D-CycE are + and ++, respectively.

phenotype is rescued even more dramatically by D-CycE, which restores a wild-type phenotype in up to 50% of the adults (Fig. 6C) and only rarely generates small-eyed flies (Fig. 6D). Rescue of the headless phenotype by CycE is not restricted to *ey-GAL4/+*; *UAS-Gsb/+* flies, but is achieved for all Pax proteins and transcription factors whose potency to interfere with *ey* function in the eye-antennal disc was tested (Table 2; Fig. 6E-G). However, in contrast to headless flies produced by Gsb, Prd, Poxm, D-Pax2 or Dac, many of which were rescued by CycE to adults that eclosed spontaneously, those generated by Mef2, Sim or Poxn were rescued at best to class IV pharates. D-Myc was not as efficient in its rescue ability (Table 2), except in the case of D-Pax2, in which nearly all flies were rescued to wild-type adults (Fig. 6H).

We conclude that developmental pathway interference through ectopic expression of transcription factors results in the inhibition of cell proliferation that is at least partially overcome by co-expression of D-Myc or D-CycE.

DISCUSSION

Expression of a transcription factor at a new location, as exemplified by homeotic mutants, is an effective, though not exclusive, way of altering the genetic program in a subpopulation of cells. To investigate how ectopic transcription factors affect a specific developmental pathway, we have examined their effects on *Drosophila* eye development after ectopic expression at an early or late stage, i.e. before or after cell proliferation. Our results demonstrate that most ectopic transcription factors, whether expressed early or late in eye-antennal discs, interfere in a detrimental way with normal development. Early ectopic expression interferes with the program of the disc primordium, by which cells prepare for proliferation, and blocks cell division. As a result, all derivatives of the eye-antennal disc fail to develop and headless flies emerge. This indicates that the gene network activated by Toy and Ey (Czerny et al., 1999; Gehring and Ikeo, 1999; Kumar and Moses, 2001) is crucial not only for the development of the eye but for that of nearly the entire head, an interpretation corroborated by our analysis of strong *ey* mutants, which also show a headless phenotype (Fig. 2E). Astonishingly, the early interference inhibiting cell division may be removed or alleviated by high levels of D-CycE or D-Myc. On the other hand, late ectopic expression of transcription factors in developing cone cells interferes with the differentiation program and causes the loss of some cone cells. Upon further reduction of D-Pax2 levels, loss of cone cells is dramatically enhanced and gives rise to a strong lensless phenotype, similar to that of *lz*-null mutants.

Our results have interesting implications for development and evolution. It appears that developmental pathways are very sensitive to the ectopic expression of transcription factors that prime different developmental programs, and react by activating latent intrinsic mechanisms that block further development. As ectopic expression of transcription factors is usually destructive for the organism, it appears that the pathways of evolutionary change are severely restricted to extremely rare instances in which an ectopic factor provides the organism with a selective advantage. Alternatively, if ectopic factors exert no effect, their newly acquired expression pattern is presumably rapidly lost. We discuss possible additional implications of our results, many of which are speculative, but may help us to understand new mechanisms of development and evolution.

Developmental pathway interference is a general mechanism that restricts evolutionary pathways

Our attempts to alter the program of eye development by ectopic transcription factors suggest that the probability of

successfully changing it is very low because of developmental pathway interference. Although our results are restricted to eye development, we found the same interference effects with the pathways of larval muscle (H. D. and M. N., unpublished) and male accessory gland development (L. Xue and M. N., unpublished). Remarkably, interference with and inhibition of a developmental pathway is not restricted to ectopic transcription factors of other developmental pathways, but may also occur with new hybrid transcription factors that could originate by independent assortment of domains from different transcription factors (Fig. 5), a process through which gene networks are thought to be expanded and modified during evolution (Frigerio et al., 1986; Noll, 1993).

Ectopic expression of transcription factors is not the only mechanism that may alter the genetic program of an organism. For example, the ectopic activation of signaling pathways (Flores et al., 2000; Kumar and Moses, 2001; Freeman, 1997) or the ectopic expression of any protein that is able to produce a change in activity or level of one or several transcription factors may be equally effective. However, all these cases ultimately affect the activity of transcription factors and hence are equivalent to their ectopic expression studied here. Therefore, we consider our findings to be of general significance.

Interference depends on time and level of ectopic transcription factor

Ectopic expression of a transcription factor does not always interfere with development and may have no detectable effect. We assume that in these cases, the activities of the target genes of the factor are not significantly changed because additional factors required for such a change are absent, or because the ectopic factor affects its targets in the same way as the normal program. Similarly, ectopic expression does not interfere maximally with the program during all developmental stages, but its maximum effect is restricted to a relatively short period. It is attractive to speculate that during this period, determinative processes occur that restrict the developmental fate. Thus, interference with the Ey/Toy pathway produces headless flies only during a very short period at the onset of Ey expression in the eye-antennal disc primordium (Table 1). The ectopic expression of Poxn in cone cell precursors interferes with eye development at a much later time during cone cell differentiation. In this case, interference also appears to be restricted to a short time interval, as ectopic expression of Poxn through the *sev* enhancer, whose activity precedes that of the *spa* enhancer, interferes strongly with cone cell differentiation, whereas regulation by the *spa* enhancer causes little interference.

In addition, interference depends on the concentration of the ectopic transcription factor. Surprisingly, it also depends on the relative level of the ectopic transcription factor to that of the transcription factor activating the normal developmental pathway. Thus, overexpression of Ey before cell proliferation or of D-Pax2 during cone cell differentiation can overcome the respective interference almost completely. This finding indicates that the new program interferes in a competitive manner with the normal pathway. This interference may result in an inhibition of cell division or in abnormal differentiation. In either case, if the interference is sufficiently strong, i.e. if at the crucial time the ectopic factor completely overrides the factor that promotes normal development, the cells eventually disappear, presumably because apoptosis is induced (Bonini and Fortini, 1999). Thus, crucial aspects of the mechanism of interference appear to be the block in cell division and/or the induction of apoptosis.

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 development through ectopic transcription factors (Fig. 2E). Therefore, one of the earliest functions of *ey* is the activation of the cell division cycle with which ectopic transcription factors interfere. As interference is restricted to a short phenocritical period during the second half of embryogenesis, we conclude that Ey primes cell division in eye-antennal development about 24 hours before eye-antennal disc cells divide in first instar larvae.

Sharp boundaries between domains of expression may be the consequence of mutual developmental pathway interference

We expect that many transcription factors will be restricted to their realms and thus give rise to sharp boundaries between their domains of expression. Indeed, such boundaries are abundant during development and may result from the necessity of the factors to avoid interference with the developmental program of the adjacent domain. Examples of early developmental pathway boundaries established at the blastoderm stage are those between transcription factors encoded by pair-rule genes. A classical example is the ubiquitous expression of *fushi tarazu* (*ftz*) in *Hs-ftz* embryos at this stage, which results in the loss of those epidermal structures in which *ftz* is normally not expressed (Struhl, 1985). Consistent with the cuticular phenotype of *Hs-ftz* embryos, we assume that interference results in a block of cell division followed by apoptosis. Similar to our observations, interference is restricted to a very short time interval around cellular blastoderm (Struhl, 1985). It should be noted that a complementary situation arises in ftz^{-} mutants in which absence of Ftz protein results in developmental pathway interference in those regions where it is normally required (Struhl, 1985). Hence, absence of a transcription factor may also lead to developmental pathway interference if it results in an undefined developmental program. This is not the case, for example, in homeotic loss-of-function mutants. Other examples of sharp boundaries are observed in mouse embryos between different types of paired domain transcription factors, such as between Pax2 and Pax6 in the developing eye (Torres et al., 1996), or between Pax3 and Pax6 (Goulding et al., 1993) and between Pax2 and Pax6 (Schwarz et al., 1999) 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 eclose (<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 eclose spontaneously (about 10%) or to pharates 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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