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Functional analysis of the chicken δ1-*crystallin* enhancer activity in *Drosophila* reveals remarkable evolutionary conservation between chicken and fly

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Summary

Functional conservation of enhancers among evolutionarily diverged organisms is a powerful way to identify basic regulatory circuits and key developmental regulators. This is especially applicable to Crystallin genes. Despite unexpected heterogeneity and diversity in their DNA sequences, many studies have revealed that most of the Crystallin genes are regulated by a relatively small set of developmentally important transcription factors. The chicken *δ1-crystallin* is one of the best-characterized Crystallin genes. Its lens-specific regulation is governed by a 30 bp long DC5 fragment present in the third intron of the gene. DC5 contains PAX6 and SOX2 binding sites, and its activity depends on the cooperative binding of these two transcription factors. To test the idea that *Pax6* and *Sox2*, together with the DC5 enhancer, could form a basic regulatory circuit functional in distantly related animals, we introduced the DC5 fragment into *Drosophila* and studied its activation pattern and regulation. The results show that the DC5 enhancer is not only active in the compound eye but, remarkably, is specifically active in those cells responsible for Crystallin secretion in *Drosophila*, i.e. the cone cells. However, regulation of the DC5 enhancer is carried out not by *Pax6*, but by *Pax2* (*D-Pax2*; *shaven* – FlyBase) in combination with the *Sox2* homologue *SoxN*. Both proteins (D-PAX2 and SOXN) bind cooperatively to the DC5 fragment and activate the enhancer synergistically. As PAX6 and PAX2 proteins derive from the same ancestor, we propose that during evolution *Pax6* function in vertebrate lens development was retained by *Pax2* in *Drosophila*.

Key words: Crystallin, Enhancer conservation, *Pax6*, *Pax2*, *Sox2*, *SoxN*, *Drosophila*

Introduction

Many transcription factors that play key developmental roles are functionally conserved among distantly related organisms. Several examples have been reported in which one transcription factor can substitute for an orthologous counterpart in an evolutionarily diverged organism and rescue the defects associated with loss-of-function mutations. In other cases, transcription factors that control appendages or organ identity induce homologous organ development when expressed ectopically in an evolutionarily unrelated organism (e.g. Acampora et al., 2000; Halder et al., 1995 and Onuma et al., 2002). As these transcription factors are supposed to interact with different regulatory sequences to control the expression of multiple genes, the principle that arises from these results is that, to some extent, the regulatory sequences to which they bind should be also conserved in different phyla. Hence, the study of functional conservation of enhancers in a wide range of organisms is a reliable way to identify basic regulatory circuits.

Lens development has long been used as a model system for the study of tissue differentiation. Complex eyes with lenses exist in a wide range of animals, from vertebrates to invertebrates (Tomarev and Piatigorsky, 1996; Piatigorsky, 2003). In all cases, the lenses are transparent structures, the primary function of which is to refract light on to the retina. Lens differentiation is accompanied by the expression of several lens-specific genes, such as Crystallins, which encode structural proteins responsible for the transparent and refractive properties of the lens. The Crystallin proteins accumulate in the lenses and can account for 80-90% of the water-soluble protein content of the lens (Piatigorsky, 2003). Although all Crystallin proteins fulfil a similar function, comparative analysis has revealed an unexpected heterogeneity and diversity among the members of this family (Piatigorsky, 1993). The vertebrate Crystallins can be divided into two groups: ubiquitous Crystallins and taxon-specific Crystallins. The former are present in all major vertebrate lenses and show sequence similarity to stress proteins. The latter are restricted to certain taxonomic groups or species, and are related or identical to metabolic enzymes (Wistow and Piatigorsky, 1998; Piatigorsky, 2003). The invertebrate Crystallins have not been studied so extensively. Nevertheless, some of them have been

molecularly characterized and also show sequence similarity to metabolic enzymes (Piatigorsky, 2003). In *Drosophila*, one of the Crystallin proteins from the corneal lens was isolated and showed to be related to insect cuticle proteins (Komori et al., 1992; Janssens and Gehring, 1999).

Despite Crystallin heterogeneity, many studies have shown that most Crystallin genes are regulated by a small set of evolutionarily conserved transcription factors (Cvekl and Piatigorsky, 1996). The chicken *δ1-crystallin* is one of the best-characterized Crystallin genes. It is a taxon-specific Crystallin present in birds and reptiles (Wistow and Piatigorsky, 1987). Its lens-specific regulation is under the control of the DC5 fragment located within the 1 kb-long intronic enhancer. The DC5 fragment is just 30 bp long and contains both a PAX6 and a SOX2 binding site. Extensive in-vitro and in-vivo analyses have demonstrated that DC5 activity depends on the synergistic action and cooperative binding of PAX6 and SOX2 to the DC5 fragment (Kamachi et al., 2001). PAX6 is a member of the Pax protein family and contains two DNA-binding domains: a paired domain and a homeodomain. It is considered a master regulator of eye development (Gehring and Ikeo, 1999) and a key transcription factor in vertebrate lens development (Cvekl and Piatigorsky, 1996). In addition, it is also necessary for the development of the nose and pancreas and parts of the central nervous system. SOX2 is a member of the Group B1 subfamily of Sox transcription factors. SOX proteins bind to DNA in a sequence-specific manner by means of a high-mobility group (HMG) domain, and are involved in a variety of developmental processes, either activating or repressing specific target genes through interaction with different partner proteins (Kamachi et al., 2000; Wilson and Koopman, 2002). It has been proposed that partnering with co-DNA-binding factors is the mechanism SOX proteins use to distinguish their regulatory targets and act in a cell-type-specific fashion (Kamachi et al., 2000).

To test the idea that *Pax6* and *Sox2*, together with the DC5 enhancer, could form a basic regulatory circuit functional in distantly related animals, we introduced the DC5 enhancer into *Drosophila* and studied its activation pattern and regulation in the eye field. The *Drosophila* compound eye is made up of approximately 800 identical units called ommatidia. Each ommatidium contains a set of retinal cells, consisting of eight photoreceptors, 12 accessory cells and a lens. The lens has two parts: the corneal lens and the crystalline cone, and it accumulates Crystallin proteins secreted by the underlying cone cells and primary pigment cells (Wolff and Ready, 1993).

The results presented in this report show that the DC5 enhancer is not only active in the *Drosophila* compound eye but, remarkably, is specifically active in those cells responsible for Crystallin secretion, i.e. the cone cells. However, regulation of the DC5 enhancer is carried out not by *Pax6*, but by *Pax2* (*D-Pax2*; *shaven* – FlyBase) in combination with the *Sox2* homologue *SoxNeuro* (*SoxN*). PAX2 and PAX6 are closely related Pax proteins. Both proteins recognize almost identical consensus sequences with their paired domain (Czerny and Busslinger, 1995), and to some extent are functionally interchangeable in *Drosophila* (Kozmik et al., 2003).

These results suggest that, despite evident anatomical, developmental and physiological differences between the vertebrate eye and the *Drosophila* compound eye, basic genetic regulatory circuits involved in the development of these two evolutionarily diverged eyes have been largely conserved.

Materials and methods

Fly strains and clonal analysis

Flies were reared on standard medium at 25°C. The following lines were used alone or in combinations: dpp^{blink}-Gal4 (Staehling-Hampton and Hoffmann, 1994), UAS-*ey* (Halder et al., 1995), UAS-*toy* (Czerny et al., 1999), UAS-*SoxN* (this study), UAS-*D* (Sànchez-Soriano and Russell, 2000), UAS-*DsRed1* (this study), spa^{pol}, $SoxN^{U6-35}$ (Overton et al., 2002), *w*; $P(w^{+mC})$36F *FRT40A* (Bloomington Stock Center), *ey-flp* (Newsome et al., 2000).

SoxN loss-of-function clones were generated by Flp-mediated mitotic recombination using the null allele $SoxN^{U6-35}$ and the FLP/FRT system (Xu and Rubin, 1993). Flies with the genotype *w*; $SoxN^{U6-35}$ *FRT40A/CyO* were crossed to *w Bac([P3-DsR]-DC5(8x)wt)*; $P(w^{+mC})$36F *FRT40A*/$P(w^{+mC})$36F *FRT40A*; *ey-flp/ey-flp* and the offspring analysed to detect mutant clones in the compound eye.

DNA constructs and transgenic flies

Wild-type (wt) and mutant forms (M4 and M7) of the octamerized DC5 enhancer were cloned as *Eco*RI fragments into the vector pSLfaGFPfa, upstream of a *Drosophila* basal promotor (*hsp27* heat-shock promoter) and the EGFP reporter gene. The vector pSLfaGFPfa was constructed by cloning the fragment *Eco*RI-*hsp27*-EGFP-SV40polyA-*Hin*dIII into the plasmid pSLfa1180fa (Horn and Wimmer, 2000). The 'cassettes' containing DC5(8x)-*hsp27*-EGFP-SV40polyA were then isolated as *Asc*I fragments and cloned into the *piggyBac* transposon derived vector pBac[3xP3-DsRedaf]. This vector contains the *DsRed1* gene, under the control of the artificial 3xP3 eye promoter, as a transgenesis marker (Horn et al., 2002). The resulting plasmids pBac[P3-DsR]-DC5(8x)wt, pBac[P3-DsR]-DC5(8x)M4 and pBac[P3-DsR]-DC5(8x)M7 were used to generate *Drosophila* transgenic lines by germline transformation in yw^{1118}. The EGFP modified version, containing the Drosocrystallin signal peptide in its N-terminus (SP+EGFP), was obtained by standard PCR techniques. The wild-type EGFP was then substituted by its modified version within the plasmid pBac[P3-DsR]-DC5(8x)wt, and the resulting plasmid pBac[P3-DsR]-DC5(8x)wt-SP+EGFP used for germline transformation in *Drosophila*. The SME enhancer (a minimal *D-Pax2* eye-specific enhancer) (Flores et al., 2000) was amplified by PCR and cloned as an *Eco*RI fragment into the vector pSLfaRFPfa. This vector is similar to pSLfaGFPfa but contains mRFP (Campbell et al., 2002) instead of EGFP. The 'cassette' SME-*hsp27*-mRFP-SV40polyA was isolated as an *Eco*RI (partial digestion)-*Asc*I fragment and used to substitute the 3xP3-basal promoter-EGFP-SV40polyA 'cassette' present in the *Hermes* transposon derived vector pHer[3xP3-EGFPaf] (Horn et al., 2000), giving rise to the plasmid pHer[SME-mRFPaf]. Then, the 'cassette' DC5(8x)wt-*hsp27*-EGFP-SV40polyA was cloned as an *Asc*I fragment into the vector pHer[SME-mRFPaf], and the resulting plasmid pHer[SME-mRFP]-DC5(8x)wt used for germline transformation. The transgenic flies harbouring this construct express mRFP under the control of the SME enhancer and EGFP under the control of the DC5(8x) enhancer. A DNA fragment containing the promoter and the 5′ untranslated region of *SoxN* (from –2939 to +869. P_{SoxN}) was amplified by PCR and cloned as an *Eco*RI-*Nco*I fragment into the vector pSLfaGFPfa. The use of the *Nco*I site eliminates the *hsp27* basal promoter present in pSLfaGFPfa. Then the 'cassette' containing P_{SoxN}-EGFP-SV40polyA was isolated as an *Asc*I fragment and cloned into the vector pHer[SME-mRFPaf]. The resulting plasmid pHer[SME-mRFP]-P_{SoxN} was used for germline transformation in *Drosophila*. The transgenic flies containing this construct express mRFP under the control of the SME enhancer and EGFP under the control of the P_{SoxN} promoter. Detailed descriptions of the primers used for the cloning procedure described above are available upon request.

The UAS-*SoxN* construct was made as follows: a genomic P1 clone containing the complete *SoxN* gene was digested with *Nhe*I, filled in with Klenow and digested with *Not*I, and introduced into pCasper cut

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with *Eco*RI/klenow and *Not*I. The resulting construct contains the complete *SoxN* open reading frame flanked by 0.75 kbp 5′UTR.

For the UAS-*DsRed1* construct, a *Bam*HI-*Xho*I fragment containing the *DsRed1* gene was isolated and cloned into pUAST cut with *Bgl*II and *Xho*I.

Cryosections

Young adult flies (within 1 day after hatching), expressing fluorescence proteins in the compound eyes, were beheaded under anaesthesia. Heads were then imbedded in OCT compound (Miles) and frozen in liquid N_2. Sections of 10 µm were cut with a cryostat, dried at 50°C for 2 minutes and directly analysed with a fluorescence microscope.

Protein expression and DNA binding assays

The following recombinant proteins were produced and purified from *Escherichia coli* according to manufacturer's instructions. The amino acids involved in each construct are shown in brackets: EY-PD (37-166), TOY-PD (29-156) and D-PAX2-PD (175-302) were tagged with 6xHis at its N-terminus (Qiagen). D (100-382) and SOXN (158-261) were expressed as N-terminal GST fusion proteins (Amersham Pharmacia Biotech).

Gel mobility shift assays were used to study protein-DNA interactions. Probes containing wild-type or mutant DC5 sequences, binding reactions and gel electrophoresis conditions are described in Kamachi et al. (Kamachi et al., 2001).

Cell culture and transfection assays

Reporter vectors were constructed by cloning the wild-type and mutant DC5(8x) enhancers in the plasmid pLacZH. pLacZH is a modified version of pLacZi (Clontech), in which the yeast minimal promoter P_{CYC1} was replaced by the *Drosophila* minimal promoter *hsp27*. The expression vectors were constructed by cloning the cDNAs of the tested genes (*ey*, *toy*, *D-Pax2*, *D* and *SoxN*) into the vector pAc5.1B/V5His (Invitrogen) under the control of the constitutively active *actin5c* promoter. For reporter gene assays 1.5×10^6 *Drosophila* Schneider 2 cells were transfected with a total of 200 ng of DNA (20 ng reporter plasmid, 5 ng of a plasmid constitutively expressing firefly luciferase, 50 ng of expression plasmids and pAc5.1B/V5His to bring total DNA to 200 ng) using the Effectene Transfection Reagent (Qiagen).

Results

In order to test the activity of the chicken DC5 enhancer in *Drosophila*, we made use of recently described new transformation vectors based on the *piggyBac* and *Hermes* transposons (Horn and Wimmer, 2000; Horn et al., 2002). These vectors contain fluorescence markers (RFP and EGFP) that allow detection of transgenic flies and analysis of enhancer activity in living adult flies without histological dissection.

DC5 is active in the cone cells of the adult *Drosophila* compound eye and its precursors in the larval eye imaginal disc

The DC5 fragment contains elements sufficient to elicit lens-specific enhancer action in its multimeric form in chicken cells (Kamachi and Kondoh, 1993). Accordingly, an octamerized version of the DC5 enhancer was introduced into *Drosophila* upstream of the reporter gene EGFP and a minimal promoter (Fig. 1B). DsRed1 under the control of the synthetic promoter 3xP3 (Horn et al., 2000) was used as a marker for transgenesis. Two mutant forms of the enhancer representing the most stringent SOX2 site and PAX6 site mutations (M4 and M7,

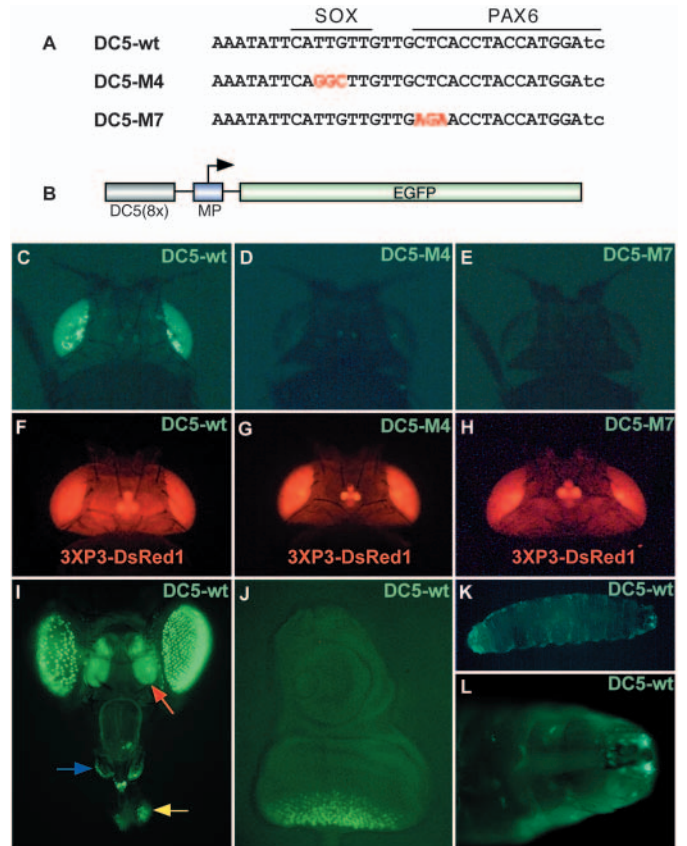

Fig. 1. Functional analysis of the chicken DC5(8x) enhancer in *Drosophila*. (A) Wild-type and mutant sequences of the DC5 enhancer used in this study. SOX and PAX6 binding sites are indicated. Altered nucleotides are shown in red. (B) Scheme of the construct used to test the functionality of the DC5 enhancer in *Drosophila*. The octamerized DC5 enhancer was cloned upstream of a minimal promoter (MP) and the EGFP reporter gene. (C-I) Activity pattern of the DC5(8x) enhancer in the adult *Drosophila* head. Enhancer activity was detected in the compound eye when the wild-type sequence was used (C). The mutant M4 and M7 enhancers failed to drive EGFP expression (D,E), although the transgenesis marker used (3xP3-DsRed1) was equally expressed in the three cases (F,G,H), indicating that the chromosomal insertion point of the different constructs did not affect enhancer functionality. (I) Enhancer activity is also detected in the adult antenna (red arrow), the maxillary palps (blue arrow) and the labial palps (yellow arrow). (J-L) During larval development, the DC5(8x) enhancer is active in the eye imaginal disc (J) and in Bolwig's organ (K,L).

respectively) (Fig. 1A) (Kamachi and Kondoh, 1993) were also octamerized and introduced in the same way. In these transgenic flies, the wild-type DC5 enhancer was active in the adult compound eye, but not in the ocelli (Fig. 1C). Furthermore, the two mutant forms of DC5 showed no enhancer activity (Fig. 1D,E), suggesting that the integrity of both binding sites is also important for the enhancer action in *Drosophila*. Additional EGFP expression was also detected in the antennae of young flies and in the adult mouthparts (the labial and maxillary palps) (Fig. 1I). The activity of the DC5 enhancer was traced back during *Drosophila* development. Enhancer action was first detected in the eye imaginal disc during the third instar larva, in cells posterior to the

morphogenetic furrow (MF) (Fig. 1J). Expression was also detected in the larval visual system (Bolwig's organ) (Fig. 1K,L). In the adult compound eye, DC5 activity was found in non-neuronal cells under the lenses, in a position resembling the one occupied by the cone cells. In order to determine the exact nature of these cells, we compared the EGFP signal with the pattern of mRFP expressed under the control of the SME enhancer. SME is specifically active in cone cells and corresponds to the minimal eye-specific enhancer of the *Drosophila Pax2* homologue *D-Pax2* (Flores et al., 2000). The results (Fig. 2A-J) showed a clear co-localization of both fluorescent proteins, which unambiguously identified the cells in which DC5 is active as cone cells. The fact that EGFP signal was detected neither in the corneal lens nor in the crystalline cone illustrates how these lens structures are formed in *Drosophila*. By contrast to vertebrates, where lens formation is a cellular process, in *Drosophila* the lenses are secreted by the cone cells and the primary pigment cells into an acellular space on the top of the ommatidia. To verify this, we modified the EGFP protein, inserting a signal peptide in its N-terminus, and analysed the location of the fluorescent protein in transgenic flies expressing the signal-peptide-tagged EGFP under the control of the DC5 enhancer. The results showed that the EGFP signal localized in the compound eye lenses, corroborating the identity of the cells responsive to the DC5 enhancer as lens secreting cells (Fig. 2K,L).

Drosophila Pax6/2 and *Sox2* homologues can cooperatively bind to the DC5 sequence

The exactly functional conservation of DC5 activity in evolutionarily highly diverged animals such as *Drosophila* and chicken, raises the question of whether there is an underlying molecular mechanism conserved in both organisms. DC5 activity in chicken was shown to be due to the cooperative binding and synergistic action of PAX6 and SOX2. Therefore, we started our analysis checking whether these transcription factors could also be involved in DC5 regulation in *Drosophila*. *Drosophila* contains two *Pax6* homologues, *eyeless* (*ey*) and *twin of eyeless* (*toy*) (Quiring et al., 1994; Czerny et al., 1999), and two *Sox2* homologues, *Dichaete* (*D*) and *SoxNeuro* (*SoxN*) (Nambu and Nambu, 1996; Russell et al., 1996; Crèmazy et al., 2000). Two additional *Pax6*-like genes are also present in *Drosophila*: *eyegone* and *twin of eyegone* (*twin of eyg* – FlyBase) (Jang et al., 2003). However, their paired domains are truncated, and hence were not considered in our analysis. By contrast, we did analyse *Drosophila Pax2* homologue (*D-Pax2*) (Fu and Noll, 1997), because it contains a complete paired domain and its expression pattern overlaps with the activity profile displayed by the DC5 enhancer (Fig. 2E-J). We expressed and purified from *E. coli* chimeric proteins containing the paired domain of EY, TOY and D-PAX2 (tagged with a Histidine tail) and the HMG DNA-binding domain of D and SOXN [fused to glutathione transferase (GST)] (Fig. 3A). The ability of these recombinant proteins to bind to the monomeric DC5 sequence was tested using gel mobility shift assays. The results (Fig. 3B,C) show that the five proteins can bind to DC5. Binding was prevented when the mutant forms of DC5 were used (data not shown). In the presence of DC5-M4 (SOX2 site mutant) binding of GST-D and GST-SOXN was abolished, whereas His-EY, His-TOY and His-D-PAX2 considerably reduced their binding capabilities in the presence of DC5-M7 (PAX6 site mutant). These results are in agreement with the in-vivo data presented above, concerning the lack of functionality of the mutant forms of the enhancer in *Drosophila*. We also compared the binding affinity displayed by His-EY, His-TOY and His-D-PAX2 paired domains in their binding to the monomeric DC5 enhancer (Fig. 3C). The results showed that His-D-PAX2 bound efficiently to the DC5 sequence, and that its binding affinity was at least ten times higher than the one displayed by His-EY and His-TOY under the same conditions. Indeed, a retardation band was detected in the presence of 0.5 ng of His-D-PAX2, whereas 5 ng of His-TOY were required to detect a band with approximately the same intensity. In the case of His-EY, even a higher amount of

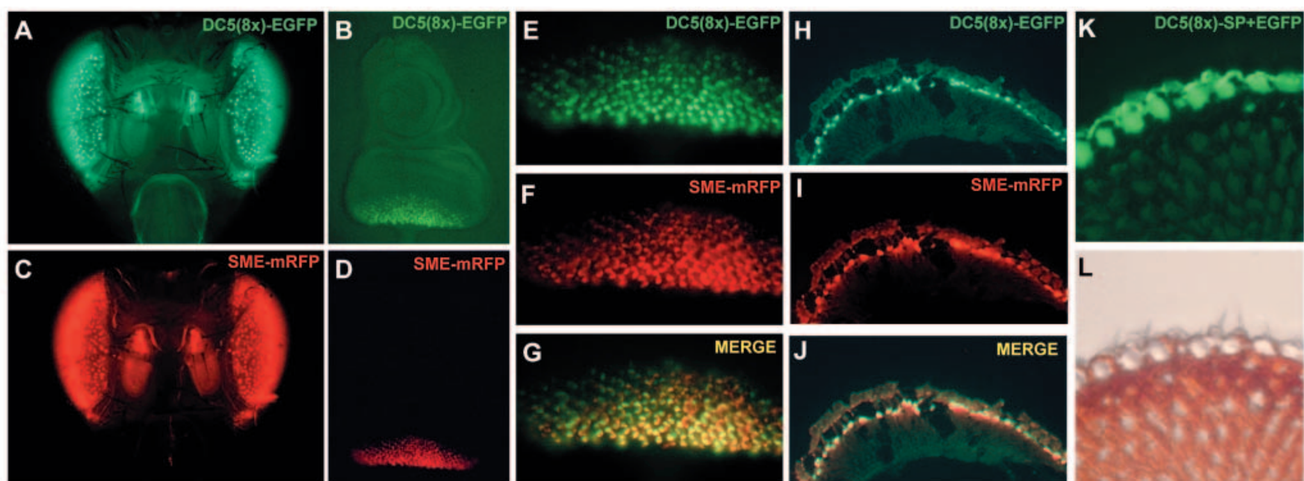

Fig. 2. The cells responsive to DC5 activity in *Drosophila* are the cone cells. The activity pattern of DC5(8x) in the adult compound eye (A) and in the larval eye imaginal disc (B) was compared with the activity pattern of the cone-cell-specific enhancer SME (C,D). SME is the minimal eye-specific enhancer of *D-Pax2* (Flores et al., 2000). Fluorescence microscopy indicates that both activity patterns are identical, as shown in preparations of the larval eye imaginal disc (E,F,G) and in cryosections of the adult compound eye (H,I,J). When a signal-peptide-tagged EGFP was used as a reporter for the enhancer activity, the fluorescence signal was detected in the lenses of the compound eye (K). This experiment was done in a w^+ genetic background to keep the ommatidium structure unaltered (L).

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protein would have been needed to match this result. Therefore, we can consider the PAX6 binding site present in the DC5 sequence as a putative PAX2 binding site, and from now on it will be called a PAX6/2 binding site.

We then addressed the question of whether the proteins could bind to the enhancer in a cooperative way, as it was described for the chicken counterparts (Fig. 3D,E,F). When PAX6/2 and SOX2 homologues – i.e. EY, TOY, D-PAX2, D and SOXN – were included in binding reactions, a slowly migrating band was detected, which probably represents the ternary complex DC5-PAX6/2-SOX2. This band appeared even at low concentrations of PAX6/2 and SOX2 homologues, concentrations at which either of them alone failed to form a retardation band with DC5. For instance, 2.5 ng of GST-D were needed to form a clear retardation band with DC5, whereas at least 5 ng of His-EY were necessary to do the same thing (Fig. 3D). However, the triple complex band was detected when 0.5 ng of GST-D were combined with 1 ng of His-EY. Furthermore, in the presence of 2.5 ng of GST-D a clear retardation band was formed with DC5 (red asterisk in Fig. 3D). Upon addition of increasing concentrations of His-EY, the band migrated more slowly (triple complex) and became more intense (blue asterisk in Fig. 3D). All these data indicate that GST-D and His-EY cooperate in their binding to DC5. The same can be applied to the rest of combinations of PAX6/2 and SOX2 homologues (Fig. 3D,E,F).

In summary, we can conclude that in vitro *Drosophila* PAX6/2 and SOX2 homologues can bind cooperatively to the DC5 enhancer.

Drosophila Pax6/2 homologues activate DC5 in vivo when *Sox2* homologues are co-expressed

We then examined whether the cooperative binding to DC5 detected among the studied proteins resulted in a synergistic

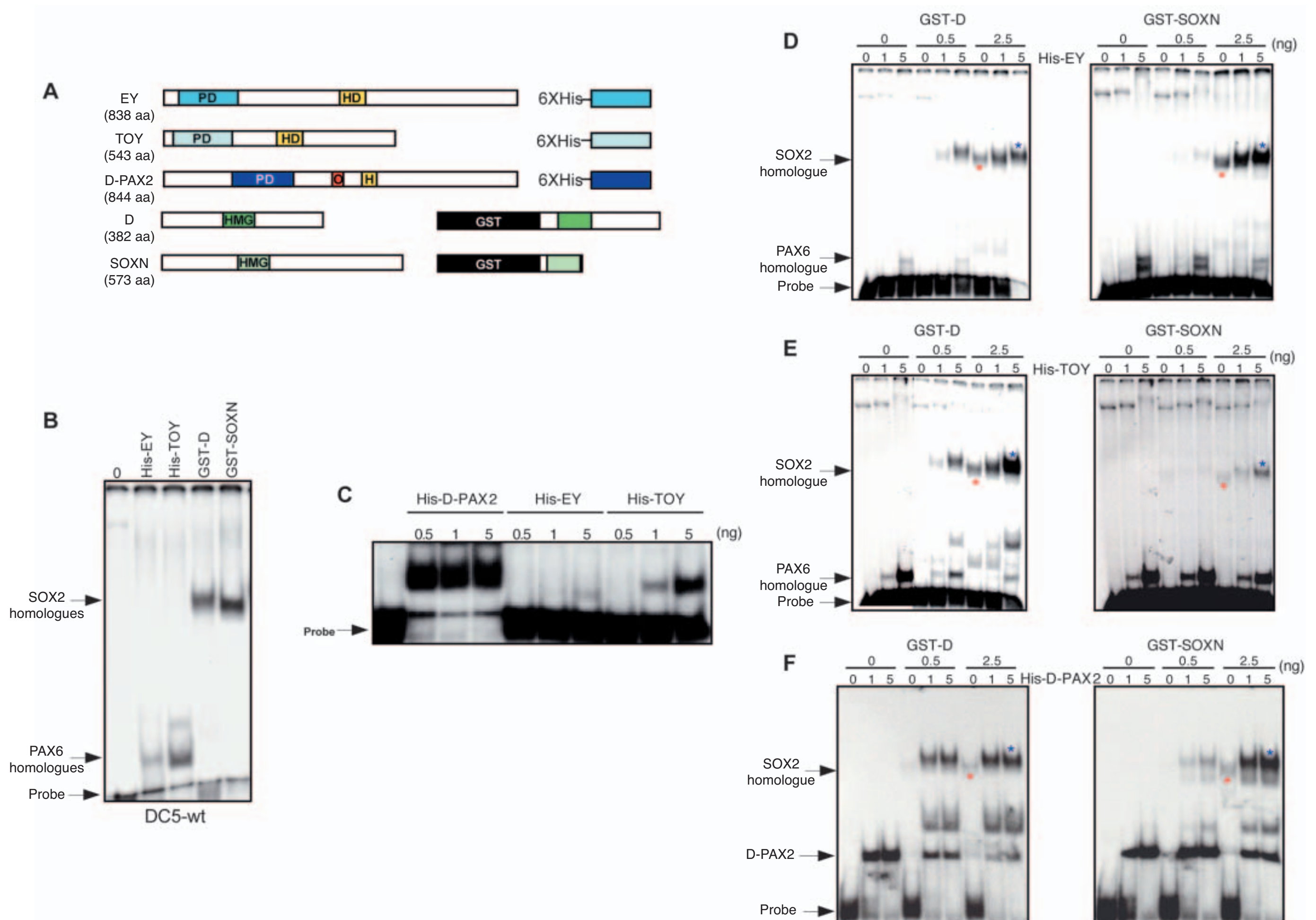

Fig. 3. Cooperative binding of the *Drosophila* PAX6/2 and SOX2 homologues to the DC5 sequence in vitro. (A) Schemes of the *Drosophila* PAX6, PAX2 and SOX2 homologues and their variants with different molecular tags. DNA-binding domains are indicated: paired domain (PD), homeodomain (HD), N-terminal portion of the homeodomain (H) and high mobility group domain (HMG). The octapeptide sequence (O) present in D-PAX2 is also shown. (B) Binding of the *Drosophila* PAX6 and SOX2 homologues to the wild-type monomeric DC5 sequence. Five nanograms of the tagged variants of the different proteins were used in gel mobility shift assays. (C) Comparison of the binding affinities of D-PAX2-PD, EY-PD and TOY-PD for the monomeric DC5 sequence. (D,E,F) Cooperative binding of *Drosophila* PAX6/2 and SOX2 homologues to the wild-type monomeric DC5 sequence. Combinations of the different tagged variants were included in the binding reaction, and their ability to cooperatively bind to the DC5 sequence was analysed by gel mobility shift assays. In all the cases, the results were similar. The duplex complex SOX2 homologue-DC5 (red asterisks) migrated more slowly and became more intense upon addition of increasing amounts of the PAX6/2 homologues, giving rise to the triple complex SOX2 homologue-PAX6/2 homologue-DC5 (blue asterisks).

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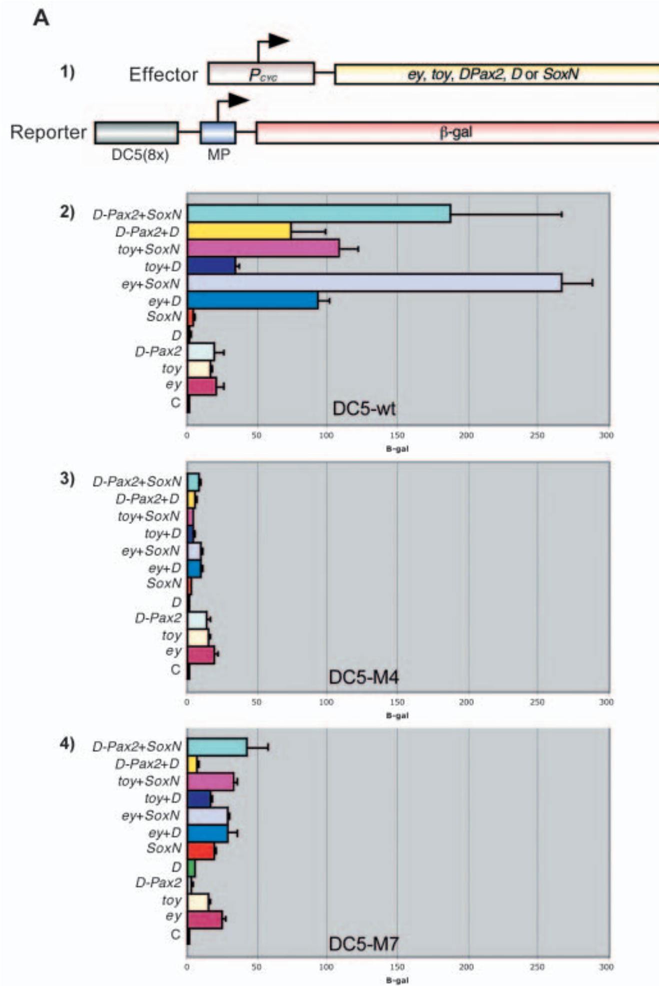

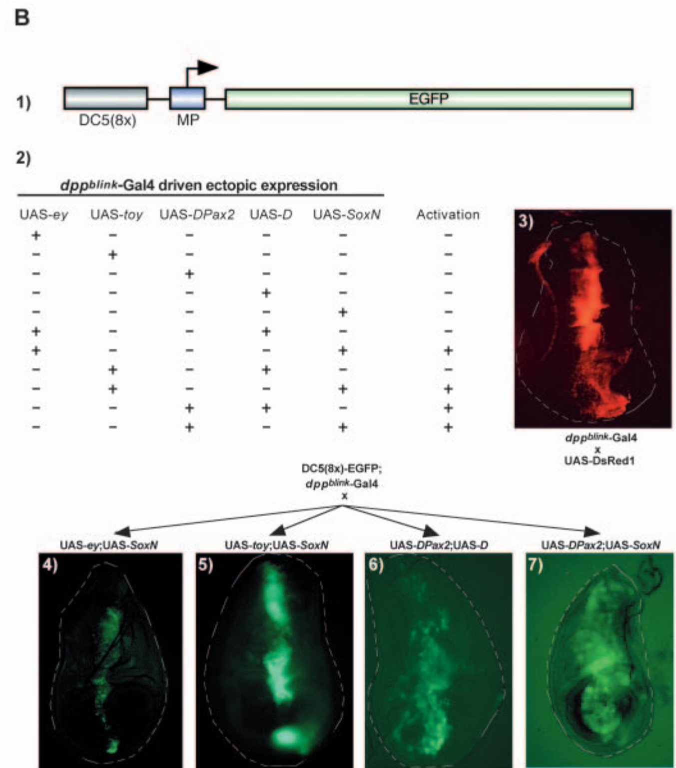

Fig. 4. Synergistic activation of the DC5(8x) enhancer in vivo. (A) Cell culture co-transfection assays in Schneider 2 cells. Wild-type and mutant DC5 enhancers were tested for activation by exogenous *Drosophila Pax6*, *Pax2* and *Sox2* homologues. The structure of the reporter and effector plasmids is shown (A1). β-gal activity of the reporter vector co-transfected with the empty effector vector was taken as 1 (column C). (B) Ectopic activation in *Drosophila* tissue. Wild-type DC5(8x) enhancer was tested for activation in the wing imaginal disc by ectopic expression of *Drosophila Pax6*, *Pax2* and *Sox2* homologues.

activation of the enhancer ex vivo. To test this possibility, we carried out cell culture co-transfection assays using *Drosophila* Schneider 2 (S2) cells. A reporter plasmid containing the DC5 enhancer upstream of the *β-galactosidase* gene was co-transfected with effector vectors expressing *Drosophila Pax6*, *Pax2* and *Sox2* homologues (Fig. 4A1). When these genes were separately expressed, the enhancer was only very modestly activated (Fig. 4A2). High-level activation was detected only when combinations of *Pax6/2* and *Sox2* homologues were co-transfected at the same time, and activation of the DC5 enhancer reached the highest level when *ey*, *toy* or *D-Pax2* were co-expressed with *SoxN* (Fig. 4A2). When the mutant forms of the enhancer were used, the activation levels remained almost basal, even when combinations of *Pax6/2* and *Sox2* homologues were co-expressed (Fig. 4A3,A4).

The results shown above support the model proposed for the regulation of DC5 in chicken, and indicate that the synergistic action of *Drosophila Pax6/2* and *Sox2* homologues is also able to efficiently activate the DC5 enhancer. Nevertheless, these results do not determine to what extent the five *Drosophila* genes are really involved in this process, and if the activation levels detected in S2 cells ex vivo are sufficient to achieve activation of the DC5 enhancer in *Drosophila* tissue in vivo. In order to corroborate our previous findings, we carried out ectopic expression of the candidate genes in *Drosophila* imaginal discs and checked for activation of the DC5 enhancer (Fig. 4B). We used the UAS/Gal4 system (Brand and Perrimon, 1993) to ectopically express the five genes under the control of the dpp^{blink}-Gal4 driver. The dpp^{blink} enhancer specifically drives expression of the yeast Gal4 transcription factor in the anterior-posterior compartment boundary of the imaginal discs at the third larval stage (Fig. 4B3). When each gene was separately expressed, no DC5 enhancer activation was detected (Fig. 4B2). Combinations of *ey* and *D*, or *toy* and *D*, also failed to activate the DC5 enhancer. However, when *SoxN* was co-expressed with *ey*, *toy* or *D-Pax2* a clear GFP expression band appeared in the anterior-posterior border of the wing imaginal disc (Fig. 4B4,B5,B7). Interestingly, *D-Pax2* could synergistically activate the enhancer in the presence of *D* (Fig. 4B6), whereas both *ey* and *toy* failed to do so. Probably, this is a consequence of the different binding affinities to the DC5 sequence displayed by the paired domains of the proteins encoded by those genes, and suggests that in vivo the triple complex D-PAX2-D-DC5 can form due to the higher affinity of the D-PAX2 paired domain for the PAX2/6 binding site present in the DC5 enhancer.

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DC5 activation in the *Drosophila* compound eye is attained by synergism of *D-Pax2* and *SoxN*

The data presented above indicate that several combinations of transcription factors can be responsible for activating the DC5 enhancer in vivo in the *Drosophila* compound eye, e.g. *ey*/*SoxN*, *toy*/*SoxN*, *D-Pax2*/*D* and *D-Pax2*/*SoxN* (Fig. 4B4,B5,B6,B7). However, a major constraint to their effective involvement in this process is their expression pattern. The expression of *Drosophila Pax6* homologues in the adult compound eye is controversial and has not been firmly demonstrated. Furthermore, their expression in the third instar eye imaginal disc during larval development is restricted to undifferentiated cells anterior to the MF, whereas the DC5 enhancer is active posterior to the MF in cone cell precursors (Fig. 2). By contrast, *D-Pax2* expression in cone cell precursors of the eye imaginal disc during the 3rd instar larval stage has been well documented (Fu and Noll, 1997). As revealed by the activity of its minimal eye-specific enhancer SME, *D-Pax2* is expressed not only in those cone cells precursors (Fig. 2F), but also in the cone cells of the adult compound eye (Fig. 2C,I). This expression pattern overlaps with the activity profile displayed by the DC5 enhancer (Fig. 2E,H).

D and *SoxN* expression in the eye imaginal disc has also been described. *D* is expressed anterior to the MF, along the ventro-lateral region of the eye-antennal disc (Mukherjee et al., 2000), in a domain where the DC5 enhancer is not active. However, immunostaining reveals expression of *SoxN* in cells posterior to the MF (Crémazy et al., 2001), in the same domain in which the DC5 enhancer is active. Expression of *D* and *SoxN* in the adult compound eye has not been described. However, we have found that a 3.4 kb DNA fragment containing the promoter and the 5′ untranslated region of *SoxN* (P_{SoxN}) (Fig. 5A) harbours regulatory sequences that recapitulate *SoxN* expression in the eye imaginal disc during larval development (Fig. 5C). These sequences drive expression of a reporter gene in cells posterior to the MF, which, by comparison to the SME enhancer activity pattern, were identified as cone cells (Fig. 5D,G,H). The P_{SoxN} fragment also drove expression of a reporter gene in the adult compound eye (Fig. 5B).

In conclusion, as only *D-Pax2* and *SoxN* show an expression profile coincident with the activity pattern of the DC5 enhancer, we favour the combination of these two transcription factors as the tandem responsible for the activity of the enhancer in the *Drosophila* compound eye. To unambiguously demonstrate this hypothesis, we analysed the activity of the DC5 enhancer in *D-Pax2* and *SoxN* loss-of-function situations. *spa^pol^* is a *Drosophila* mutant characterized by the lack of *D-Pax2* expression in cone cells and primary pigment cells of developing larval and pupal eye discs (Fu and Noll, 1997). The mutant fly is homozygous viable, but the compound eye shows a severe disruption of the ommatidia structure that gives rise to a rough eye phenotype. When the construct containing the DC5 enhancer was introduced into the *Drosophila spa^pol^* mutant, no expression of the reporter gene (EGFP) was detected in the adult compound eye (Fig. 6A), although the cone cells were still present, as revealed by the action of the cone-cell specific enhancer SME (Fig. 6B) (see also Fig. 6 in Fu and Noll, 1997). In the case of *SoxN*, all the available mutants were embryonic lethal. In order to analyse the effect of *SoxN* depletion on the activity of the DC5 enhancer, we generated loss-of-function clones in the adult compound eye using the yeast FLP/FRT system (Xu and Rubin, 1993). As displayed in Fig. 6E, we induced site-specific mitotic recombination between the mutant chromosome arm (blue line) and the wild-type chromosome arm (red line), using the *eyeless* enhancer to drive flip recombinase expression. As this enhancer is specifically active in the developing eye (Hauck et al., 1999; Newsome et al., 2000), the homozygous mutant clones are only induced in the compound eye, allowing the mosaic fly to survive. Under white light, the clonal mutant tissue was identified by a white coloration (red arrow in Fig. 6C), whereas the homozygous or heterozygous wild-type tissue displayed a red or orange coloration, respectively (black arrows in Fig. 6C). The mutant clones were more readily identified with the use of UV light (red arrow in Fig. 6D). The red and orange of the wild-type tissue did not completely mask the

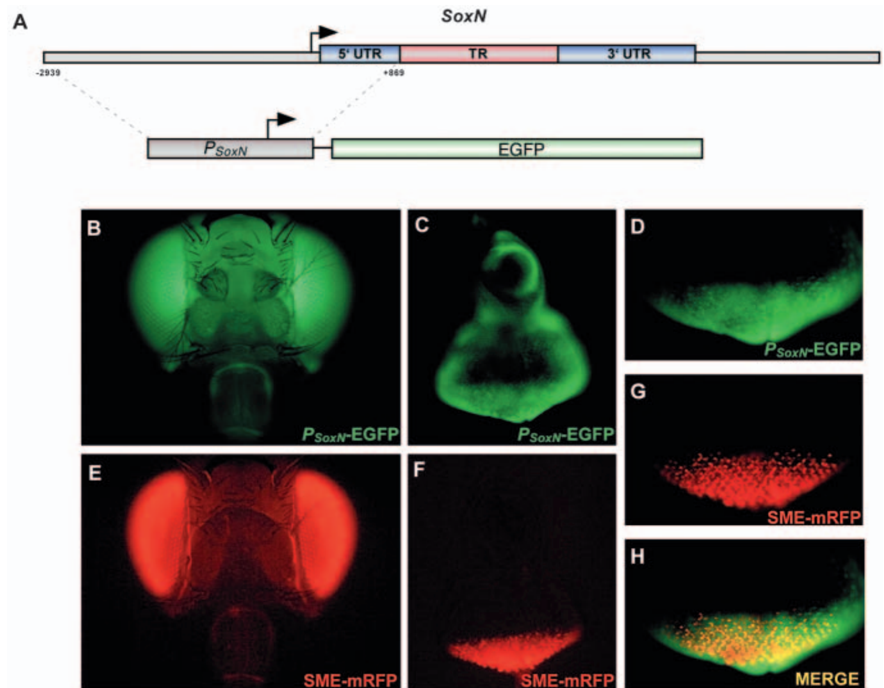

Fig. 5. *D-Pax2* and *SoxN* are expressed in the *Drosophila* compound eye and in the eye imaginal disc. (A) Genomic map of the *SoxN* locus and structure of the construct used to trace *SoxN* expression. The intronless *SoxN* gene consists of a translated region (TR) flanked by extensive 5′ and 3′ untranslated regions (5′ UTR and 3′ UTR, respectively). Regulatory sequences important for *SoxN* expression in the adult compound eye (B) and in the eye imaginal disc (C) are present in the promoter and the 5′ untranslated region of *SoxN* (from –2939 to +869. P_{SoxN}). *D-Pax2* expression was monitored using its minimal eye-specific enhancer SME. This enhancer is active in the cone cells of the adult compound eye (E) and their precursors in the eye imaginal disc (F). Comparison of both expression patterns in the eye imaginal disc shows that they are coincident (D,G,H).

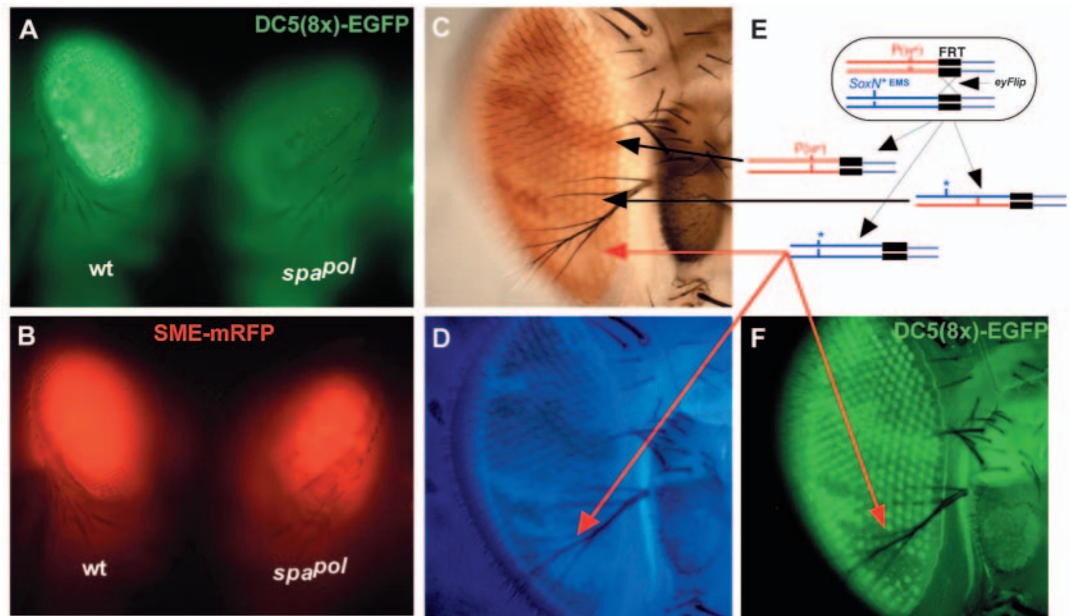

Fig. 6. DC5(8x) activity in the *Drosophila* compound eye is abolished by depletion of either *D-Pax2* or *SoxN*. (A) The DC5(8x) enhancer is inactive in *Drosophila* spa^{pol} mutant. This mutant is characterized by the absence of *D-Pax2* expression in cone cells and primary pigment cells. Nevertheless, cone cells do still form in spa^{pol} mutant, as shown by means of the cone-cell-specific enhancer SME (B). (C-F) *SoxN* depletion was studied using the clonal analysis technique. Mutant clones in the adult compound eye were generated using the strategy depicted in panel E. $SoxN^{-}$ clones (red arrows in C,D,F) were identified by the lack of red pigment (w^{-}) under white illumination (C). The use of UV light facilitated the identification of the mutant tissue (D). The activity of the DC5(8x) enhancer is abolished in the $SoxN^{-}$ clones (F).

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EGFP signal induced by the activity of the DC5 enhancer, and clear EGFP spots were visible at the top of every illuminated ommatidium when the objective was focused at the proper level (Fig. 6F). However, no EGFP signal was detected in the colourless mutant tissue, indicating that *SoxN* depletion in the compound eye abolishes the activity of the DC5 enhancer (Fig. 6F). In summary, the results obtained with the loss-of-function experiments corroborate our previous conclusions and indicate that, in wild type, DC5 enhancer activity in the *Drosophila* compound eye is due to the cooperative action of the transcription factors D-PAX2 and SOXN.

Discussion

A conserved regulatory circuit involved in lens development

The *δ1-crystallin* gene is an early marker for differentiating lens cells in chicken. The extensive work carried out by Kondoh and collaborators have led to the finding that this gene is regulated by a 1 kb-long enhancer located in the third intron. Within this enhancer, a small DNA fragment of only 30 bp (DC5 fragment) confers lens-specificity. The DC5 fragment contains two binding sites, a PAX6 binding site and a SOX1/2/3 (Group B1 SOX proteins, SOX2 being the major player) binding site, and the cooperative binding of these transcription factors to the DC5 sequence activates the enhancer in a synergistic fashion (Kamachi and Kondoh, 1993; Kamachi et al., 1995, 2000, 2001; Kondoh et al., 2004).

The simplicity of the DC5 fragment, the well-characterized nature of its transcription factor binding sites, and the fact that *Pax6* and *Sox2* are important developmental regulators conserved in evolution, prompted us to consider these three elements (DC5 sequence, *Pax6* and *Sox2*) as part of a conserved regulatory circuit involved in lens development. To test this idea, we performed a functional enhancer test and introduced the DC5 fragment into a distantly related organism, *D. melanogaster*. Functional conservation of enhancer elements has been previously reported. Exchanges of Hox and *Pax6/eyeless* enhancer elements between flies, worms and vertebrates gave rise to expression patterns that were characterized as homologous (Streit et al., 2002; Frasch et al., 1995; Xu et al., 1999). In other cases, enhancer elements from a variety of *D. melanogaster* neuronal and muscular genes failed to activate the expression of a reporter gene in the homologous cell types in *Caenorhabditis elegans* (Ruvinsky and Ruvkun, 2003). These various outcomes are probably due to differences in the evolutionary pressure exerted on different enhancers according to their developmental roles. However, these results emphasize the importance of this test when the result is positive, meaning that the functional conservation of an enhancer is a reliable way to identify basic regulatory circuits.

The fruit fly and the chicken are separated by hundreds of millions of years of evolution, and their visual organs reflect this evolutionary distance at the anatomical, developmental and physiological level. Even the eye lenses, although fulfilling a similar function, are formed differently. In chicken the lenses are cellular structures, whereas in *Drosophila* they are secreted into an acellular space by the cone cells and the primary pigment cells. The introduction of the chicken DC5 enhancer into *Drosophila* had a remarkable effect. Not only was the DC5 enhancer active in the *Drosophila* compound eye, but also it was specifically active in the cells that are in part responsible for lens secretion in *Drosophila*, i.e. the cone cells. The experiment was done with an octamerized version of the DC5 enhancer to augment the sensitivity of the system. Actually, when a single copy of the DC5 enhancer was used, no activity was detected in the *Drosophila* compound eye (data not shown). This suggests that although lens-specificity is retained by DC5 in *Drosophila*, additional sequences have to be present to provide full activity to the enhancer.

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DC5 regulation is under the control of *D-Pax2* and *SoxN* in *Drosophila*

Once demonstrated that the DC5 enhancer is active in *Drosophila*, we focused our attention on finding out whether the other two elements of the regulatory circuit were also conserved, i.e. whether DC5 activity in *Drosophila* was due to the synergistic action of *Pax6* and *Sox2*. Gel mobility shift assays showed that *Drosophila* PAX6 homologues (EY and TOY) and SOX2 homologues (D and SOXN) could bind cooperatively to the DC5 sequence. Interestingly, the PAX6 paired domain was sufficient for DNA binding and cooperation with D and SOXN. Cell culture co-transfection assays and ectopic activation of the DC5 enhancer corroborated these findings, and showed that the DC5 enhancer was synergistically activated upon co-expression of *Drosophila Pax6* (*ey* or *toy*) and *SoxN*. However, an important constraint to the real involvement of these transcription factors in the regulation of the DC5 enhancer in vivo is their expression pattern. Whereas *SoxN* expression was detected in both the adult compound eye and in cone cell precursors of the eye imaginal disc (Fig. 5B,C), expression of *Drosophila Pax6* homologues in the adult compound eye is controversial and has not been clearly demonstrated. In the eye imaginal disc, during the third larval stage, *Drosophila Pax6* homologues are expressed in undifferentiated cells anterior to the MF, whereas DC5 is active posterior to the MF in cone cell precursors. However, *Drosophila Pax2*, a gene evolutionarily related to *Pax6*, is expressed in cone cells, primary pigment cells and bristle cells of larval and pupal eye discs (Fu and Noll, 1997). In addition, a *D-Pax2* cone-cell-specific enhancer has been characterized in the fourth intron of the gene (Fu et al., 1998; Flores et al., 2000). This enhancer (called SME) is active in cone cells of the adult compound eye and their precursors in the eye imaginal disc (Fig. 2F,I), mimicking the activity pattern of DC5. In-vitro and in-vivo studies showed that D-PAX2 could cooperate with SOXN in binding to the DC5 sequence (indeed it showed a higher affinity for DC5 than *Drosophila* PAX6 homologues), and thus activate the enhancer in a synergistic fashion. Finally, loss-of-function analysis showed conclusively that depletion of either *D-Pax2* or *SoxN* abolished DC5 activity in vivo, clearly demonstrating the involvement of these two transcription factors in the activation of the DC5 enhancer in the *Drosophila* compound eye.

It is interesting to point out that, apart from the compound eye, the DC5 enhancer is also active in other tissues of the adult fly, such as the antenna and the mouthparts (the labial and maxillary palps). It is not known whether *Drosophila Pax6*, *Pax2* and *Sox2* homologues are expressed in these tissues of the adult fly, although *D-Pax2*, *D* and *SoxN* expression has been detected in the developing antennal disc (Fu and Noll, 1997; Mukherjee et al., 2000; Crémazy et al., 2001), and *ey* has been shown to be involved in the development of the fly maxillary palps and antennae (Benassayag et al., 2003). This opens the possibility of different combinations of transcription factors taking part in the regulation of the DC5 enhancer in other tissues besides the eye, and suggests that partnering among these factors might also be used for developmental processes other than eye development. In addition, the fact that several of these combinations can effectively activate the DC5 enhancer in an ectopic situation (Fig. 4B4,B5,B6,B7) further supports this assumption. Interestingly, we could detect that the cone-cell-specific enhancer SME also drove expression of a reporter gene in the non-compound-eye tissues in which DC5 is active, namely the adult antenna and the labial and maxillary palps (data not shown). Therefore, we favour the hypothesis that *D-Pax2* is also responsible for the activity of the DC5 enhancer in these fly appendages.

Pax6 function in vertebrate lens development was taken over by *Pax2* in *Drosophila*

The results obtained with the analysis of the chicken DC5 enhancer in *Drosophila* have important evolutionary implications. They suggest that *Pax6* function in vertebrate lens development was probably retained by *Pax2* in *Drosophila*. PAX2 and PAX6 are closely related proteins. They recognize almost identical consensus sequences with their paired domains (Czerny and Busslinger, 1995) and to some extent can be considered as functional homologues in *Drosophila*, as *D-Pax2* is able to induce ectopic eyes (*Pax6* function) and *ey* and *toy* can rescue *spa*^{pol} mutation (*Pax2* function) (Kozmik et al., 2003). In agreement with this, a recently characterized cnidarian *PaxB* gene was tentatively identified as the descendant of the last common ancestor of the *Pax6* and *Pax2* genes (Kozmik et al., 2003; Gehring, 2004; Piatigorsky and Kozmik, 2004). Like PAX2, PAXB protein contains a PAX2/5/8-type paired domain and octapeptide; and, like PAX6, a complete paired-type homeodomain. As a consequence, *PaxB* is able to rescue the *Drosophila spa*^{pol} mutation (*Pax2* function) and to induce small ectopic eyes in *Drosophila* (*Pax6* function). Interestingly, *PaxB* is also able to activate the jellyfish *J3-crystallin* promoter in cell culture co-transfection assays (Kozmik et al., 2003). It is tempting to speculate that after duplication and diversification of the ancestor *PaxB-like* gene, Crystallin regulation was retained by *Pax6* in the vertebrate lineage, whereas this function was taken over by *Pax2* in *Drosophila*. We can speculate further and suggest that this functional diversification was probably due to changes in the regulatory elements of these two genes and not in their coding sequences. At present, *Pax6* and *Pax2* genes show structural differences that reflect the changes that occurred in their coding sequences during evolution. As well as the paired domain, *Pax6* contains a homeodomain; *Pax2*, however, has only part of the homeodomain and, in addition, an octapeptide sequence accompanying the paired domain. Nevertheless, as mentioned above, *Drosophila Pax6* and *Pax2* genes are functionally exchangeable and can largely substitute for some of each other's functions. The same seems to apply to the regulation of the DC5 enhancer in *Drosophila*. Both *Pax6* (*ey* and *toy*) and *D-Pax2* can activate this enhancer in vivo when they are co-expressed with *SoxN*, but the main limitation to their real involvement in DC5 regulation is their expression pattern, meaning differences in their regulatory elements.

In summary, we propose that after duplication of the *PaxB-like* ancestor, changes in the regulatory sequences determined which paralogous gene took over Crystallin regulation. Once the expression pattern of the duplicated genes diverged, changes in their coding sequences brought about the structural differences detected today, to better adjust each of the paralogous genes to its developmental role. According to our analysis, it seems that Crystallin regulation was taken over by *Pax6* in vertebrates, whereas this function was retained by *Pax2* in *Drosophila*.

Drosophila Crystallin genes under the control of the same regulatory circuit

The fact that lens-specificity of the DC5 fragment is retained in *Drosophila* suggests that a similar mechanism could be responsible for Crystallin regulation in the fruit fly. This is supported by the phenotype of the *Drosophila spa^pol^* mutant. In this mutant, as previously mentioned, *D-Pax2* expression is abolished in both the cone cells and the primary pigment cells (Fu and Noll, 1997). As a consequence, the hexagonal lattice of ommatidia is severely disrupted, giving rise to a rough eye phenotype. In *spa^pol^* most of the cone cells and many primary pigment cells are still present (Fu and Noll, 1997) and retain the ability to secrete corneal lenses and crystalline cones. However, these lens structures are frequently defective and fused or display the blueberry-eye phenotype (Fu and Noll, 1997). We think these lens defects are not a secondary effect of the disruption of the ommatidium structure, but are probably due to the absence of *D-Pax2* expression in the cone cells and primary pigment cells. Crystallin genes under the putative control of *D-Pax2* would fail to be expressed in this situation, bringing about the lens defects observed in the *Drosophila spa^pol^* mutant.

As mentioned above, *Drosophila* Crystallin proteins are secreted by cone cells and primary pigment cells, and accumulate on the top of the ommatidium forming the corneal lens and the crystalline cone. Two-dimensional gel electrophoresis has identified 14 different proteins in the crystalline cone (Tomarev and Piatigorsky, 1996) and only three proteins in the corneal lens (Komori et al., 1992). The most abundant protein in the corneal lens is Drosocrystallin, a 52 kDa protein that contributes to lens development by providing the appropriate refractive index to the corneal lens (Komori et al., 1992). Sequencing of the N-terminus end of Drosocrystallin allowed the cloning of the *drosocrystallin* (*dcy*) gene and its characterization as a member of the insect cuticular protein gene family (Janssens and Gehring, 1999). Interestingly, *dcy* expression was detected in the primary pigment cells, but not in the cone cells (Komori et al., 1992) (data not shown). As *D-Pax2* is expressed in both cell types, other regulatory differences between *dcy* and DC5 must exist to account for their distinct expression patterns. A 441 bp DNA fragment, including the promoter region of *dcy*, was shown to be sufficient to drive expression of a reporter gene into the primary pigment cells (Janssens and Gehring, 1999). We are currently dissecting this DNA fragment and investigating its regulation to find similarities and differences with DC5 regulation.

The isolation and characterization of new *Drosophila* Crystallin genes has been impaired by the heterogeneous nature of these genes. During evolution, proteins with different enzymatic activities have been recruited (or co-opted) to fulfil a Crystallin role, both in vertebrates and in invertebrates. An event common to all these Crystallin co-options has been the acquisition of highly lens-specific expression of the recruited proteins, through changes in the regulatory regions of their genes (Piatigorsky, 2003). The results presented in this report suggest that the chicken DC5 enhancer might be one of the genetic elements used throughout evolution to recruit new genes into lens development. We are currently using this information to identify new *Drosophila* Crystallin genes, and to find out whether their lens-specific expression is achieved by regulatory elements similar to the chicken DC5 enhancer.

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