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DNA-Binding Characteristics of Cnidarian Pax-C and Pax-B Proteins In Vivo and In Vitro: No Simple Relationship With the Pax-6 and Pax-2/5/8 Classes

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ABSTRACT Cnidarians are the simplest animals in which distinct eyes are present. We have previously suggested that cnidarian *Pax-Cam* might represent a precursor of the Pax-6 class. Here we show that when expressed in *Drosophila* imaginal discs, Pax-Cam chimeric proteins containing the C-terminal region of EY were capable of eye induction and driving expression of a reporter gene under the control of a known EY target (the *sine oculis* gene). Whilst these results are consistent with a Pax-6-like function for *Pax-Cam*, in band shift experiments we were unable to distinguish the DNA-binding behaviour of the Pax-Cam Paired domain from that of a second *Acropora* Pax protein, Pax-Bam. The ability of a Pax-Bam/EY chimera to also induce eye formation in leg imaginal discs, together with the in vitro data, cast doubt on previously assumed direct relationships between cnidarian Pax genes and the Pax-6 and Pax-2/5/8 classes of bilateral animals. *J. Exp. Zool. (Mol. Dev. Evol.) 299B:26–35, 2003.*

INTRODUCTION

To a surprising extent, common molecular mechanisms appear to underlie the early morphogenesis of eyes across the animal kingdom. The idea of a common molecular basis of eye-specification has its origins in the discovery that *Drosophila eyeless* (*ey*) is orthologous with the mammalian Pax-6 genes (Quiring et al., '94) and that ectopic eyes can be generated by expression of *ey* or *Pax-6* in *Drosophila* imaginal discs (Halder et al., '95). *ey* and *Pax-6* are expressed in comparable patterns in the developing CNS and eye, and loss-of-function mutations in the corresponding genes result in strikingly similar phenotypes in *Drosophila*, mouse and man (eyeless, Small eye and aniridia) (Callaerts et al., '97). Pax-6 genes have subsequently been identified in a wide variety of higher animals, and are expressed in eye primordia in animals as diverse as the squid *Loligo* (Tomarev et al., '97) and ribbonworm, *Lineus* (Loosli et al., '96). A Pax-6 gene is expressed in regenerating and adult eyes in the flatworm *Dugesia tigrina* (Callaerts et al., '99), implying that the function of this gene has been conserved throughout the Bilateria. These findings have led to the proposal that Pax-6 may be a ''master control'' gene for eye specification, and the hypothesis that vision has a single origin (Callaerts et al., '97; Gehring and Ikeo, '99). However, it is not yet clear whether true Pax-6 genes are present in non-bilateral animals, such as cnidarians.

The Cnidaria are the closest outgroup to the Bilateria (Medina et al., 2001), and occupy a key position in the evolution of complexity—they are the simplest animals at the tissue level of organization. Despite the absence of a central nervous system with which to process images, distinct eyes ranging in complexity from simple eye-spots to complex lens eyes are present in many representatives of three of the four cnidarian

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classes. The most sophisticated eyes are present in the most motile of cnidarians, the Cubozoa (box jellyfish). The cubozoan eye may consist of up to 11,000 sensory cells, and has an epidermal cornea, spherical lens and a retina with distinct sensory, pigmented and nuclear layers (Brusca and Brusca, '90). Some cnidarians, including *Hydra*, lack any obvious photoreceptors but clearly react to light (Tardent and Frei, '69), and photosensitivity is considered to be a general property of the phylum.

To better understand the evolution of the Pax gene family, we are characterizing the Pax gene complement of the coral *Acropora millepora*, which is a member of the basal cnidarian class, the Anthozoa (Bridge et al., '92; Medina et al., 2001). Members of this class lack the motile medusa (jellyfish) stage that is characteristic of the other cnidarian classes and, although anthozoans lack eyes, they display photosensitive behaviour. For example, coral polyps are extended at night and retracted during the day, and coral larvae display a variety of phototactic behaviours (reviewed in Harrison and Wallace, '90).

Pax genes are defined by the presence of a paired box, which encodes the 128AA Paired domain (PD), and fall into approximately five classes on the basis of sequence similarity and the presence/absence of other motifs such as the homeodomain (HD) and octapeptide. Although these genes have a wide variety of specialized roles, many of them function early in development in the nervous system. *Acropora* has four Pax genes (Catmull et al., '98; Miller et al., 2000), and two of these (*Pax-A* and *Pax-B*) have been cloned from several other cnidarians (Sun et al., '97; Hoshiyama et al., '98; Gröger et al., 2000; Sun et al., 2001). *Pax-A* is likely to be orthologous with *Drosophila poxneuro* (Catmull et al., '98), which was previously considered to be a highly diverged Pax-2/5/8-related gene. *Pax-D*, which is so far only known from *Acropora*, is likely to represent a precursor of the Pax-3/7 class (Miller et al., 2000). We have previously proposed that cnidarian *Pax-B* and *Pax-C* represent precursors of the Pax-2/5/8 and Pax-6 classes respectively, and have shown that the Pax-Bam Paired domain binds Pax-2/5/8 consensus sites (Miller et al., 2000). Both sequence comparisons and domain structure are broadly consistent with these proposed relationships. Pax-B proteins resemble the Pax-2/5/8 class in containing an octapeptide motif (this motif is not present in other cnidarian Pax proteins), but differ in that the former contain complete, rather than truncated, homeodomains. Consistent with a role in neural differentiation or patterning, *Pax-Cam* is expressed in presumed neurons during larval development (Miller et al., 2000). However, the notion of a simple correspondence between these cnidarian genes and Pax-6 and Pax-2/5/8 classes is controversial. One problem with the hypothesis that Pax-C represents a Pax-6 precursor is that this implies that a Pax-C gene should be involved in specifying jellyfish eyes, and to date there is no evidence that this is the case. Several groups have surveyed the Pax complements of various jellyfish (Sun et al., '97; Gröger et al., 2000; Sun et al., 2001), but genes related to *Pax-Cam* have not been detected. *Pax-B* was the only *Pax* gene detected in the hydrozoan jellyfish *Podocoryne*, and this gene may be involved in nerve cell differentiation (Gröger et al., 2000). The scyphozoan jellyfish *Chrysaora* and *Cladonema* have simple and complex lens eyes respectively; *Pax-B* has been cloned from both species, but *Pax-A* was not detected in the latter (Sun et al., '97; Sun et al., 2001).

To better understand the relationship of the cnidarian genes with the Pax-6 and Pax-2/5/8 classes, we studied the DNA-binding specificity of Pax-Cam and Pax-Bam Paired domains in vivo and in vitro. The results indicate that both Pax-Cam and Pax-Bam proteins bind to EY targets in vivo and in vitro, and thus indicate that the relationship between these cnidarian proteins and the Pax-6 and Pax-2/5/8 classes of bilateral animals is unlikely to be simple. The literature suggests that Pax gene loss may be an ongoing process within the Cnidaria. We suggest that, in non-anthozoans, Pax-B may have acquired the roles of Pax-Cam or, alternatively, that within the Anthozoa Pax-C may have arisen from a Pax-B-like ancestor to fulfill more restricted roles.

MATERIALS AND METHODS

Fly strains

Flies were reared on standard medium at 25°C. Lines used were *so-LacZ* (Cheyette et al., '94), *dpp^{blk}-GAL4*/TM6b (Staehling-Hampton et al., '94), UAS-*ey* (Halder et al., '95). Transgenic lines were generated via P-element-mediated germ line transformation; β-galactosidase assays were performed as described by Niimi et al. ('99).

Chimeric Drosophila expression constructs

Domain-swap constructs were generated in pUAST (Brand and Perrimon, '93) using the

technique of splicing by overlap extension (Clackson et al., '91). This method uses two rounds of PCR. In the first of these, products are obtained from two templates separately, using in each reaction a primer that has a region of cross-complementarity with the reciprocal template. In the second PCR stage the products of the two initial reactions are allowed to anneal and amplify. Chimeric constructs generated in this way were cloned into pBSK and the sequences of the inserts verified before subcloning into pUAST and transformation into *Drosophila*. The structures of the constructs used for *Drosophila* transformation are shown schematically in Figure 1. Swapping was achieved immediately following either the paired domain or the homeodomain. Sequences of all of the primers used and complete PCR protocols are available on request.

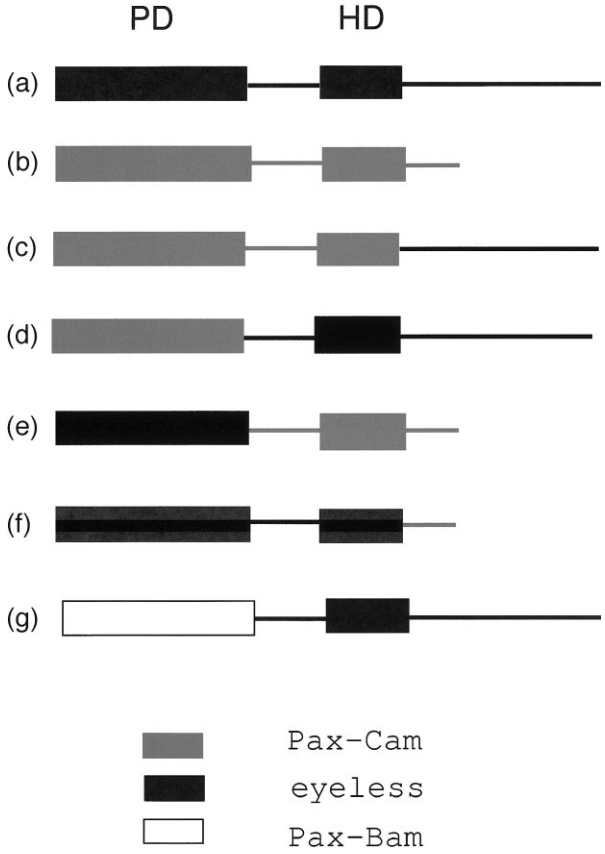

Fig. 1. Schematic representation of constructs expressed in *Drosophila* imaginal discs. (a) The EY protein, (b) Pax-Cam, (c-f) Pax-Cam/EY chimeric proteins, (g) Pax-Bam/EY chimera. PD = paired domain; HD=homeodomain. Note that the extensive region C-terminal of the PD in EY contains a well-defined transactivation domain, whereas the C-terminal region of Pax-Cam is much shorter.

Yeast one-hybrid system

The yeast one-hybrid system described by Mastick et al. ('95) was used to examine the interaction of Pax-Cam constructs with a defined EY target. The domain swap constructs described above were cloned into the activator plasmid pBM258T (Mastick et al., '95), and used in combination with the *so10*-His3 pHR307a reporter plasmid previously described (Niimi et al., '99). The basis of this system is that double transformants are only capable of growth on galactose media (galactose drives expression of the activator construct) lacking histidine if the chimeric protein constructs are capable of binding the *so10* region and activating expression of the reporter gene (*HIS3*).

Other molecular methods

Recombinant Pax-Cam and Pax-Bam Paired domains were generated via expression from pQE30 (Qiagen) constructs. These proteins were purified and gel-shift assays carried out as previously described (Miller et al., 2000). The sequences of the oligonucleotides used in the band shift experiments were: (Pax-6 consensus site) 5'-gactAGGTTCACGCTTCAGTTAGTCAGC-3'; (Pax-2/5/8 consensus site) 5'-gactCTAGTCATGCATGAGTGTTCCAGC-3'; (*so10* footprinted sequence) 5'-gactGCAAACAAGTAAAAATTAATTCCCCCTCACTGGGCACAACT-3'. In each case, the (gact) sequence shown in lower case was included to allow labeling, and was present only in the forward primer. Annealing of the forward primer with its reverse complement resulted in a double-stranded oligonucleotide with the four base single stranded extension on one end, which was then end-filled using Klenow in the presence of α-^{32}P-dATP. The underlined part of the *so10* probe is the sequence protected from nuclease digestion by EY as described in Punzo et al. (2002).

RESULTS

Expression of Pax-Cam in Drosophila imaginal discs results in a dominant negative-like phenotype

Expression of *Pax-6* genes from a variety of animals in *Drosophila* imaginal discs leads to the formation of ectopic eyes (Halder et al., '95; Glardon et al., '97; reviewed in Callaerts et al., '97). In order to test the morphogenetic properties

of *Pax-Cam*, the putative Pax-6 gene from *Acropora*, several independent *Drosophila* lines carrying the *Pax-Cam* cDNA transgene under the control of the yeast GAL4 UAS regulatory sequence were generated via P-element-mediated germ line transformation (Brand and Perrimon, '93). The UAS sequence upstream of the cDNA results in the transcription of the cDNA when yeast GAL4 is expressed in *Drosophila* cells. A UAS-*Drosophila eyeless* cDNA line was used as a positive control for the effects of induced EY expression. The UAS-*Pax-Cam* fly lines were crossed with the dpp-GAL4 driver, permitting expression of the *Pax-Cam* cDNA in the wing, leg and eye/antenna discs. In the case of the UAS-*ey* lines, GAL4-directed expression of EY in the any of these imaginal disc types resulted in ectopic eyes in the corresponding adult structure (Fig. 2a). GAL4-driven expression of Pax-Cam, however, not only did not result in ectopic eye formation (Fig. 2b), but also appeared to interfere with the development of adult structures arising from the disc in which it was expressed (wing, leg, eye). The legs were malformed and truncated, the wings did not develop correctly (Fig. 2e) and the eyes were reduced in size.

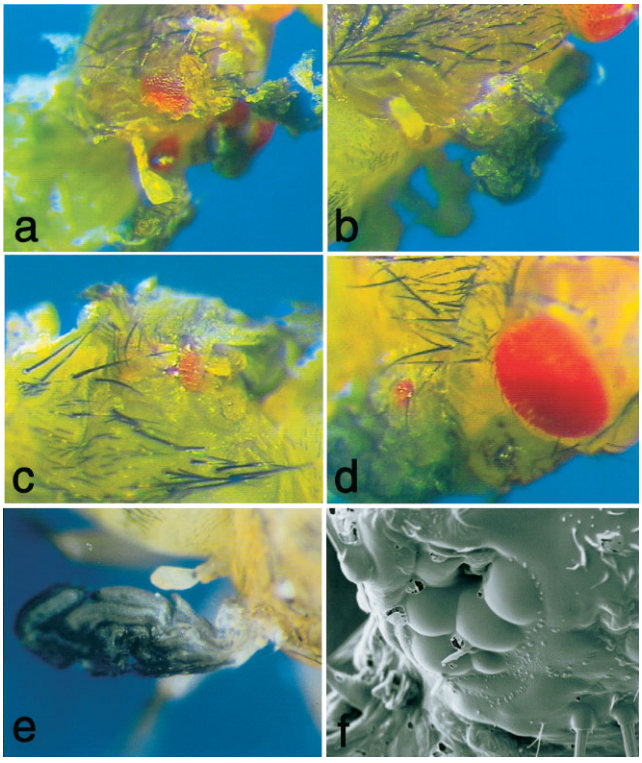

Fig. 2. Phenotypes resulting from expression of Pax-Cam constructs in *Drosophila* wing imaginal discs. The constructs shown in Fig. 1 were expressed under GAL4-UAS control in wing discs. Expression of the *eyeless* cDNA leads to the formation of ectopic eye tissue seen as the red-pigmented structure (a), whereas expression of the *Pax-Cam* cDNA does not result in eye formation (b). (c-d) Expression of chimeric constructs (c and d in Fig. 1) encoding the C-terminal region of EY result in eyes that are smaller than those induced by EY. (e) Expression of Pax-Cam in the wing disc causes severe abnormalities. (f) At the SEM level, the morphology of the eyes induced by the Pax-Cam/EY constructs can be seen to include regular ommatidia and inter-ommatidial bristles.

Chimeric Pax-Cam constructs encoding the EY C-terminal domain result in eye formation in Drosophila

Comparison of the Pax-Cam sequence with a range of PAX-6 proteins suggested that the former may lack the C-terminal transcription activation domain present in the latter, and might therefore act as a dominant negative with respect to EY targets in vivo. To test this hypothesis, a

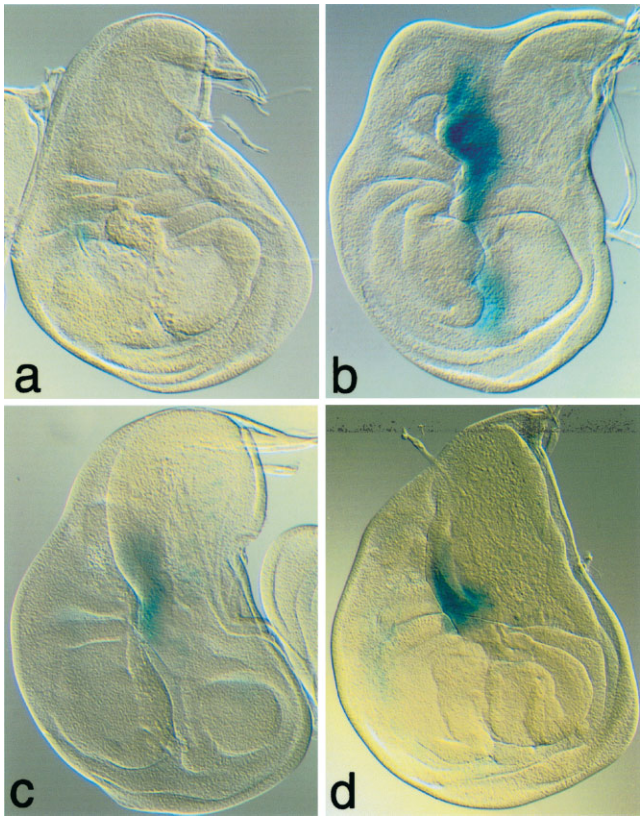

Fig. 3. β-galactosidase expression in *sine oculis (so)-lacZ* lines driven by dppGAL4-UAS Pax-Cam constructs. Wing discs are shown in which the constructs shown in Fig. 1 were expressed. (a) Pax-Cam does not induce significant so-lacZ expression, (b) positive control by misexpression of EY. (c-d) Both domain swap constructs encoding the C-terminus of EY (i.e. constructs c and d in Fig. 1) induced *so-lacZ* expression.

series of UAS-constructs were generated via splicing by overlap extension (Clackson et al., '91), in which the C-terminal region of EY was transposed onto the regions of Pax-Cam encoding the DNA-binding domains and vice versa (Fig. 1). Crosses of fly lines carrying these UAS-domain swap constructs with the dpp-GAL4 driver line showed that UAS lines in which the EY C-terminal region was present were capable of triggering ectopic eye development in the wing (Fig. 2c–d). This effect was seen with both constructs in which the Pax-Cam PD and the EY C-terminal domain were present—i.e., it was independent of the source of the homeodomain. The reciprocal constructs (i.e., EY constructs featuring the Pax-Cam C-terminal region) were incapable of inducing ectopic eyes; small amounts of ectopic red pigment were occasionally detected (not shown), but there was absolutely no evidence of developing eye structures.

Eyes induced in response to expression of Pax-Cam/EY fusion proteins were always significantly smaller than those resulting from EY misexpression (compare Fig. 2c–d with Fig. 2a), but were otherwise morphologically normal. In scanning electron micrographs, regular ommatidia and inter-ommatidal bristles are clearly visible (Fig. 2f).

Ectopic sine oculis expression is induced by Pax-Cam/EY chimeras

The *Drosophila* experiments described above implied that the Pax-Cam protein bound to EY targets in vivo. We therefore investigated the ability of the Pax-Cam PD to activate expression of a known EY target in vivo and to bind to known PD binding sites in vitro. *sine oculis* (*so*) is one of the best characterised direct targets of EY; the EY protein activates expression of *so* by binding to an eye-specific enhancer called *so10* in the *so* gene (Niimi et al., '99). Figure 3 shows LacZ staining patterns in wing discs from fly lines in which expression of the enhancer trap *so-LacZ* (Cheyette et al., '94) was driven by various EY, Pax-C or Pax-C/EY chimeric constructs. Both of the constructs in which the EY C-terminal domain was present (Fig. 3c and d) activated *so* expression, albeit at significantly lower levels than did EY (Fig. 3b). Constructs consisting of the EY PD and the Pax-Cam HD and C-terminal region did not drive significant levels of so expression (data not shown).

Pax-Cam binds to the sine oculis eye-specific enhancer region in a yeast one-hybrid system

The *Drosophila* experiments outlined above implied that Pax-Cam/EY chimeras were capable of activating expression of EY targets, such as *sine oculis*, in vivo. To better understand this interaction, we examined the ability of the corresponding chimeras to bind to the *sine oculis* eye-specific enhancer region using a yeast one-hybrid system. Yeast activator constructs expressing Pax-Cam/EY chimeras corresponding to those used in *Drosophila* were generated in pBM258T (Mastick et al., '95), and used in conjunction with the *so10-His3* reporter in pHR307a previously described (Niimi et al., '99). Results of these experiments are shown as Figure 4. When yeast strains containing both activator and reporter plasmids were plated onto media containing galactose but lacking histidine, the *HIS3* reporter was activated by EY or the Pax-Cam/EY chimeras, but not by Pax-Cam or by constructs consisting of Pax-Cam C-terminal regions fused to the EY PD or PD plus homeodomain (i.e., constructs (e) and (f) in Fig. 1). These results are consistent with the *Drosophila* data, confirming that the Pax-Cam PD binds the *sine oculis* eye-specific enhancer (*so10*) in vivo, but is incapable of activating transcription unless fused to a heterologous transactivation domain.

In vitro binding properties of Pax-Cam and Pax-Bam PDs

Previous studies (Niimi et al., '99) identified a 128 bp sequence (a *so10* subfragment) in the *so* eye-specific enhancer to which EY bound in band-shift assays. EY protects a short segment of this subfragment from nuclease digestion in DNA footprinting experiments (Punzo et al., 2002). Because the footprinted segment is likely to be an in vivo EY-binding site, we investigated the ability of the Pax-Cam PD to bind to oligonucleotides corresponding to this sequence in band-shift assays. The ability of the Pax-Cam PD to bind oligonucleotides corresponding to consensus Pax-2/5/8 and Pax-6 binding sites (Fig. 5) was also examined and, in parallel, we studied the interaction of the Pax-Bam PD with the same range of targets. In each case, the recombinant PDs bound specifically and with high apparent affinity to the labeled oligonucleotides and, although the method does not permit quantitation of the interaction, no

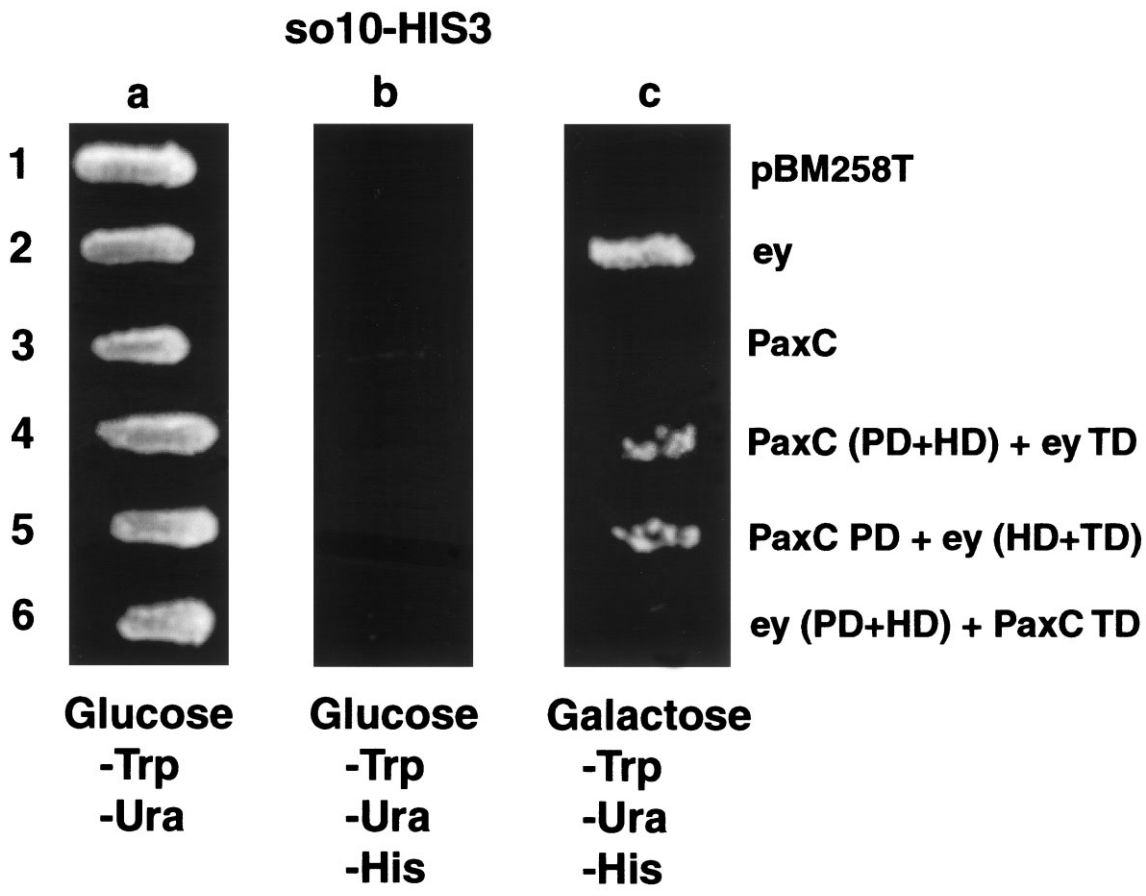

Fig. 4. Pax-Cam binds to the *sine oculis* eye-specific enhancer *so10* in a yeast one-hybrid system. Expression of the proteins indicated on the right of the figure was driven by a galactose-inducible promoter that is strongly repressed by glucose. Each panel (a, b and c) represents the same colonies plated onto different media, the composition of which is indicated at the bottom of the figure. Panel a: growth control experiment. The presence of histidine (His) allows all of the colonies to grow; the medium lacks tryptophan (Trp) and uracil (Ura) to select for maintenance of the *so10-His3* reporter and pBM258T activator plasmids respectively. Panel b:negative control experiment; no growth is observed on medium lacking histidine in the presence of glucose since activator proteins are not produced. Panel c: In the presence of galactose, yeast colonies are able to grow on media lacking histidine if the protein produced binds to the *so10* target and activates transcription of the *HIS3* reporter gene. Lane 1: pBM258T empty vector as negative control; lane 2: pBM258T Eyeless expressing vector as positive control. Lanes 3 to 6: various PaxC/EY chimeras cloned into pBM258T as indicated to the right of the figure. Lanes 4, 5 and 6 correspond to constructs c, d and f respectively in Fig 1. Note that no growth is observed with all constructs on the empty His plasmid lacking the *so10* sequence (not shown and Niimi et al., '99).

major differences were apparent between the PDs in affinity for the oligonucleotides.

Expression of a Pax-Bam/eyeless chimera in Drosophila

Because the Pax-Bam PD bound the same range of target sites in vitro as did that of Pax-Cam, the morphogenetic properties of a Pax-Bam/EY domain swap construct were examined in *Drosophila* imaginal discs. To avoid potentially complicating protein-protein interactions, and to enable direct comparison with Pax-Cam/EY phenotypes, the construct encoded only the N-terminal region and PD of Pax-Bam, and the region of EY C-terminal of the PD (Fig. 1). When expressed in either the leg or wing discs, the Pax-Bam/EY construct was able to induce ectopic eyes, albeit with lower efficiency than was the corresponding Pax-Cam/EY construct (Fig. 6; note the extremely weak wing disc phenotype). Thus, the in vivo data are consistent with the in vitro DNA-binding experiments, indicating that the PDs of both Pax-Cam and Pax-Bam bind EY targets and, in the presence of the EY C-terminal domain, can initiate compound eye morphogenesis in fly.

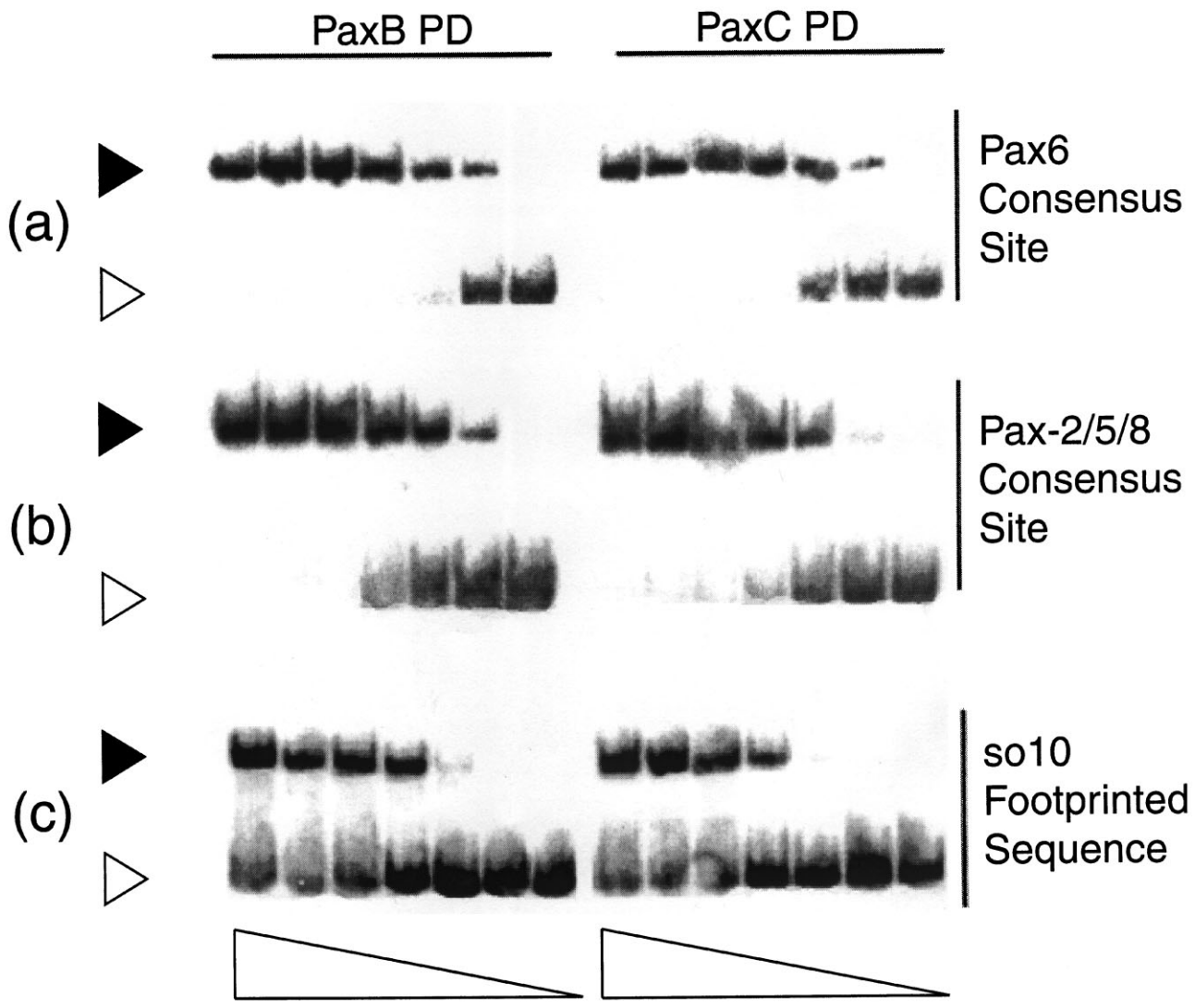

Fig. 5. DNA-binding assays using the Pax-Cam and Pax-Bam Paired domains. The binding of recombinant Pax-Cam (right panel) and Pax-Bam (left panel) PDs to (a) a consensus Pax-6 binding site (5'-AGGTTCACGCTTCAGTTAGTCAGC-3'), (b) a consensus Pax-2/5/8 binding site (5'-CTAGTCATGCATGAGTGTTCCAGC-3'), and (c) a known EY target (the *so10* oligonucleotide 5'-GCAAACAAGTAAAAATTAATTCCCCCTCACTGGGCACAACT-3') was determined by electrophoretic mobility shift assays. In each case, the concentration of oligonucleotide was held constant, and the concentration of the PD varied from 2.6×10^{-5} M to 7.9×10^{-10} M (corresponding to 8-fold dilution between lanes); the right hand lane in each case is a negative control in which no protein was added. Empty and filled triangles on the left of the figure indicate the positions corresponding to free and bound probe respectively.

DISCUSSION

Our initial goal was to test the hypothesis that *Pax-Cam* represents a precursor of the Pax-6 class by examining the morphogenetic properties of the Pax-Cam protein expressed in *Drosophila* imaginal discs. Although Pax-Cam was unable to initiate eye morphogenesis in imaginal discs, this effect appears to result from the inability of the wild-type protein to activate transcription in *Drosophila*. Expression of chimeric Pax-Cam proteins containing the C-terminal region of EY in imaginal discs resulted in eyes that were morphologically normal, but smaller than those induced by EY misexpression. The Pax-Cam/EY chimeras conferred a number of phenotypic characteristics normally associated with Pax-6 proteins, including activation of a *so-lacZ* in vivo. Similarly, Pax-Cam/EY chimeras were able to activate transcription of a *HIS3* reporter by binding to the *so10* fragment in a yeast one-hybrid system. The C-terminal region of the Pax-Cam

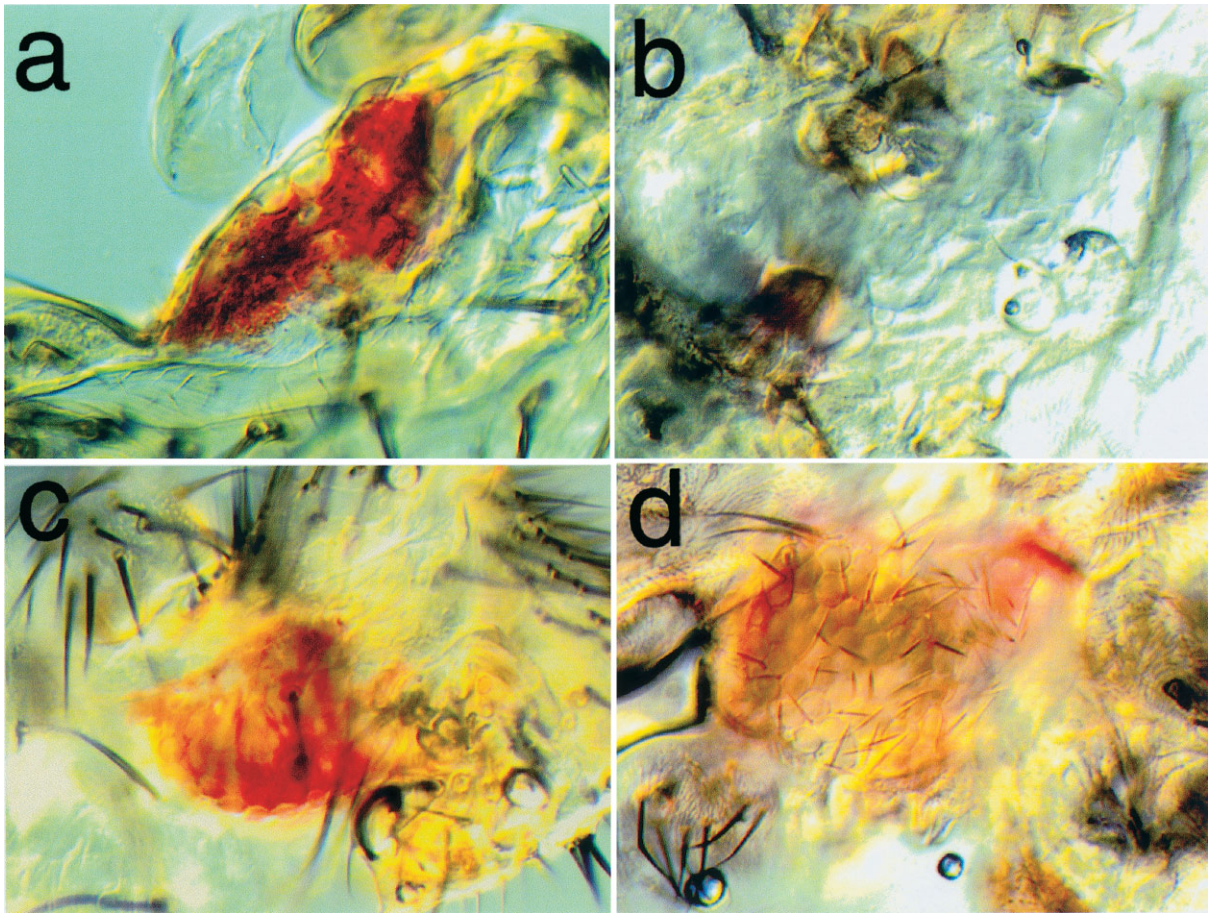

Fig. 6. Comparison of phenotypes resulting from expression of Pax-Bam/EY and Pax-Cam/EY constructs in leg and wing imaginal discs. The Pax-Bam/EY construct (shown schematically in Fig. 1g) was capable of inducing eye morphogenesis in the leg disc only (a); typical results of expressing this construct in the wing disc are shown as (b). The corresponding Pax-Cam/EY construct (shown schematically in Fig. 1d) displayed stronger morphogenetic properties in both the leg (c) and wing (d) discs.

protein is much shorter (only 81 AA residues C-terminal of the HD) than that in EY and PAX-6 proteins in general (the EY C-terminal region is 387 AA residues; that of PAX-6 is 152), and contains no obvious transcription activation domain (Czerny and Busslinger, '95; Tang et al., '98). It is therefore likely that Pax-Cam functions primarily as a transcriptional repressor, as does the PAX-6-related mammalian protein PAX-4 (Smith et al., '99).

Whilst the experiments in which Pax-C/EY chimeras were expressed in *Drosophila* support the hypothesis that Pax-Cam is a precursor of the Pax-6 class, the in vitro DNA-binding properties of a second *Acropora* Pax protein, Pax-Bam, led us to question this assumption. The Pax-Cam and Pax-Bam PDs bound the same range of sequences in vitro, including a known EY target site—a footprinted sequence in the *so* eye-specific enhancer. The PDs of the cnidarian Pax proteins appear to have relatively low DNA-binding specificities; published data for the *Cladonema* and *Chrysaora* Pax-B proteins (Sun et al., 2001) are broadly consistent with the *Acropora* data. Although the specificity of the *Acropora* Pax-A PD has not yet been determined, binding to EY targets is not a universal property of cnidarian PDs, as the *Acropora* Pax-D PD does not bind to these same sites in vitro (Nordström et al., 2003). The DNA-binding behaviour of the Pax-Bam PD in vitro led us to examine the morphogenetic properties of a Pax-Bam/EY chimera in *Drosophila*. The Pax-Bam/EY chimera was able to induce ectopic eyes in the leg disc and to a limited extent in wing discs, but with lower efficiency than was the corresponding Pax-Cam/EY construct.

Phylogenetic analyses clearly show that cnidarian Pax-B and Pax-C both belong to the Pax

supergroup which also includes the Pax-6 and Pax-2/5/8 classes (Balczarek et al., '97; Catmull et al., '98; Gröger et al., 2000; Miller et al., 2000). Although we have previously suggested otherwise (Catmull et al., '98; Miller et al., 2000), the results presented here suggest that there is unlikely to be a simple correspondence between the cnidarian Pax-B and Pax-C genes and the Pax-2/5/8 and Pax-6 classes in higher animals. The specificity associated with true Pax-6 genes presumably arose after the Cnidaria/bilateral Metazoa split. However, we cannot exclude the possibility that the specificity of PAX-B and PAX-C proteins is influenced by regions other than the PD; it is quite possible that the activity and specificity of the PD is influenced by the overall protein environment. Therefore the fact that the in vitro experiments described here were carried out with PDs alone, and the in vivo work was carried out on *Acropora* PDs in the context of the EY protein, is one major limitation in interpreting the results.

Clearly, Pax proteins are an ancient class of transcription factors (Hoshiyama et al., '98) that diversified very early in animal evolution (Miller et al., 2000). Although the possibility of undersampling cannot be discounted, accepted at face value, the Pax gene surveys that have been carried out suggest the possibility of ongoing loss of Pax genes throughout the Cnidaria. *Acropora*, a representative of the most basal class, has four Pax genes (A, B, C, D); within the Hydrozoa, two (A, B) Pax genes have been identified in *Hydra* whereas *Podocoryne* appears to have a single Pax gene (B). Similarly, within the Scyphozoa, two *Chrysaora* Pax genes (A, B) have been cloned, whereas *Cladonema* appears to have only one (B). Under the above scenario, the Pax-B and Pax-C types are likely to post-date the Cnidaria/Bilateria split; Pax-C either originated within the common cnidarian ancestor, or within the Anthozoa after the Anthozoa/Medusozoa (Hydrozoa, Scyphozoa and Cubozoa) split. Although their DNA-binding characteristics are similar, the two proteins are likely to have distinct roles—Pax-Cam presumably functions primarily as a repressor of transcription, whereas sequence comparisons imply that Pax-B proteins may be able to act either as transactivators (via the C-terminal domain) or repressors (via the octapeptide) depending on context. The presence of complete HDs in Pax-B proteins distinguishes these from the Pax-2/5/8 class proper; presumably the full HD enables Pax-B proteins also to act via their HD to regulate specific gene expression. In addition to common roles throughout the Cnidaria, the functional flexibility of Pax-B proteins may have enabled these in some cnidarians to effectively fulfill the roles of Pax-Cam in *Acropora*. Either the roles of Pax-Cam may have been subsumed by Pax-B in medusozoans (the non-anthozoan cnidarians), or Pax-Cam may be derived from a Pax-B-like precursor to fulfill more specific roles.

One prediction of the above model is that we might expect the expression patterns of Pax-B genes in non-anthozoan cnidarians to correspond to the sum of the patterns of *Pax-Cam* and *Pax-Bam* in *Acropora*. Unfortunately, expression data are available only for *Acropora Pax-Cam* and *Podocoryne Pax-BPc*. *Pax-Cam* has a very specific pattern of expression, in a subset of presumed neurons in the planula larva (Miller et al., 2000). At the same stage in *Podocoryne*, *Pax-BPc* is expressed throughout the entire ectoderm (Gröger et al., 2000). In *Podocoryne* polyps, *Pax-BPc* expression is restricted to ectodermal cells that are either interstitial cells or neurons (or both) and in medusae, the (endodermal) pattern of *Pax-BPc* expression is again consistent with a role in nerve cell differentiation (Gröger et al., 2000). Whilst these data are consistent with common functions of *Pax-BPc* and *Pax-Cam* in the nervous system, and suggest that the former may fulfill multiple roles, expression data for more Pax genes in a variety of cnidarians (particularly for *Pax-Bam*) are clearly required to test the hypothesis outlined above.

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