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Role of Pax Genes in Eye Evolution: A Cnidarian *PaxB* Gene Uniting Pax2 and Pax6 Functions

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Summary

***PaxB* from *Tripedalia cystophora*, a cubomedusan jellyfish possessing complex eyes (ocelli), was characterized. *PaxB*, the only Pax gene found in this cnidarian, is expressed in the larva, retina, lens, and statocyst. PaxB contains a Pax2/5/8-type paired domain and octapeptide, but a Pax6 *prd*-type homeodomain. Pax2/5/8-like properties of PaxB include a DNA binding specificity of the paired domain, activation and inhibitory domains, and the ability to rescue *spa^{pol}*, a *Drosophila Pax2* eye mutant. Like Pax6, PaxB activates jellyfish crystallin and *Drosophila* rhodopsin *rh6* promoters and induces small ectopic eyes in *Drosophila*. *Pax6* has been considered a "master" control gene for eye development. Our data suggest that the ancestor of jellyfish PaxB, a PaxB-like protein, was the primordial Pax protein in eye evolution and that Pax6-like genes evolved in triploblasts after separation from Cnidaria, raising the possibility that cnidarian and sophisticated triploblastic eyes arose independently.**

Introduction

Pax transcription factors are characterized by their DNA binding paired domain (Bopp et al., 1986; Treisman et al., 1991) and are associated with numerous developmental and disease processes (Chi and Epstein, 2002). Pax proteins have been grouped into four subfamilies, two of which include a second DNA binding domain, the *paired* (*prd*)-type homeodomain (Noll, 1993).

The well-characterized *Pax6* has been considered a universal "master" control gene (Gehring and Ikeo, 1999; Gehring, 2002) for the morphologically distinct eye types generated in evolution (Land and Nilsson, 2002). Heterozygous mutations in the human *PAX6* gene (*Aniridia*) result in the partial or complete absence of the iris, whereas heterozygous *Pax6* mutant mice (*Sey*) have small eyes and homozygous mutants lack eyes altogether (for review, see Hanson and van Heyningen, 1995). Reduced eyes also result from mutations in the *Drosophila Pax6* homolog *eyeless* (*ey*; Quiring et al., 1994). Remarkably, ectopic expression of *ey* (Halder et al., 1995) or *twin of eyeless* (*toy*), a second *Pax6* homolog in *Drosophila* (Czerny et al., 1999), induces ectopic eyes. *Pax6* activates the rhodopsin genes in *Drosophila* (Sheng et al., 1997; Papatsenko et al., 2001) and the lens crystallin genes of vertebrates (Cvekl and Piatigorsky, 1996; Duncan et al., 2004).

The *Pax2/5/8* subfamily comprises a single *D-Pax2* gene in *Drosophila* (Czerny et al., 1997; Fu and Noll, 1997) and three genes (*Pax2*, *Pax5*, and *Pax8*) in mammals, which arose by duplications at the onset of the vertebrate lineage (Pfeffer et al., 1998). *D-Pax2* has been implicated in development of ommatidial cone and pigment cells (Fu and Noll, 1997) and mechanosensory bristles (Fu et al., 1998; Kavaler et al., 1999). *Pax2* deficiency in mice results in kidney, eye, and inner ear defects (Torres et al., 1996). *Pax2* cooperates with *Pax5* in the development of the midbrain and cerebellum (Urbánek et al., 1997; Schwarz et al., 1997), *Pax5* is essential for brain patterning and B-lymphopoiesis in mammals (Urbánek et al., 1994; Nutt et al., 1999; Rolink et al., 1999), and *Pax8*-deficient mice display thyroid gland dysgenesis (Mansouri et al., 1998). *Pax2* and *Pax8* are partially redundant in kidney organogenesis (Bouchard et al., 2002).

The present investigation focuses on the cubomedusan jellyfish *Tripedalia cystophora*, a member of the ancient Cnidaria comprising the anthozoans and medusazoans (Collins, 2002; Galliot and Schmid, 2002). The anthozoans (corals) have four Pax genes (PaxA, B, C, and D); PaxB has been considered most closely related to Pax2/5/8 and PaxC to Pax6 (Miller et al., 2000). However, others have shown PaxC to be more closely related to PaxA and PaxB (Gröger et al., 2000; Sun et al., 2001), a conclusion consistent also with the phylogenetic tree obtained by Miller et al. (2000). The *PaxB* gene in Cnidaria (Sun et al., 1997, 2001; Gröger et al., 2000; Miller et al., 2000) and Porifera (sponges; Hoshiyama et al., 1998) encodes a Pax2-like paired domain and octapeptide, and a *prd*-type homeodomain. Although anthozoans lack eyes, the cubomedusan, *Tripedalia*, has well-developed eyes (ocelli) with striking similarities to vertebrate eyes (Piatigorsky et al., 1989). Their retinas have ciliated photoreceptors rather than the rhabdomeric photoreceptors used by most invertebrates (see Eakin, 1979), and their cellular lenses accumulate crystallins (Piatigorsky et al., 1989, 1993, 2001).

In the present investigation, we show that *Tripedalia* has only the *PaxB* gene that is expressed in swimming larvae and in the lens, retina, and statocyst of adult rhopalia. While the studies cited above have indicated a structural homology to Pax2/5/8 and Pax6, we demonstrate that this ancient transcriptional activator is a functional hybrid of the Pax2/5/8 and Pax6 subfamilies, and

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provide compelling evidence that the *J3-crystallin* gene is a natural target of PaxB. Finally, we show that overexpression of *PaxB* under the appropriate enhancer can both rescue the *spa*^{pol} phenotype and generate ectopic eyes in *Drosophila*. Our analysis suggests that a PaxB-like protein was the primordial Pax protein of eye evolution and regulator of crystallin gene expression, and that Pax6 genes evolved independently in Bilateria after their separation from Cnidaria.

Results

Isolation and Phylogenetic Relationship of *Tripedalia PaxB*

Multiple alignments of the *Tripedalia* PaxB sequence, derived from an isolated cDNA, with mouse and *Drosophila* Pax2 and Pax6 suggest that PaxB is a hybrid of the Pax2/5/8 and Pax4/6 or Pax3/7 subfamilies (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/5/5/773/DC1). As in other cnidarians (Sun et al., 1997, 2001; Gröger et al., 2000), PaxB has a Pax2-like paired domain (with 82% and 75% identity to mammalian Pax2 and Pax6) and a Pax2-like octapeptide not present in Pax6. Moreover, the three amino acids at the positions critical for the DNA binding specificity of paired domains (Czerny and Busslinger, 1995) differ from those present in Pax6 (highlighted in red in Supplemental Figure S1) and are identical to those characteristic for Pax2/5/8. However, the homeodomain of PaxB is much more closely related to that of Pax3/7 and Pax6 (with 60% and 55% identity) than to a similar region of Pax2 exhibiting about 32% similarity (Supplemental Figure S1). Finally, phylogenetic tree analysis based on paired domains showed that *Tripedalia* PaxB clusters with the Pax2/5/8 subfamily (data not shown), and that PaxB of the scyphozoan jellyfish *Chrysaora quinquecirrha* (CqPaxB; Sun et al., 1997) is its closest known relative.

PaxB Expression during the Life Cycle and in the Rhopalium of *Tripedalia*

We used RT-PCR to examine *PaxB*, *J1A-*, *J1B-*, *J1C-*, and *J3-crystallin*, and *jRXR* expression in larvae, polyps, and adult rhopalia of *Tripedalia* (Figure 1). The rhopalia of adult jellyfish were excised, and the PCR products were normalized with respect to those of rRNA (see Experimental Procedures). Expression of the *J1-* and *J3-crystallin* and *PaxB* genes was detected in rhopalia as well as larvae (Figure 1A). *PaxB* was more highly expressed in larvae than rhopalia. *PaxB* transcripts were detected in the lens of the complex eye by whole-mount in situ hybridization (not shown), and in the lens and retina of both the large and small ocelli (Figure 1B, cf. panels A and C with panels B and D) and in the statocyst (Figure 1B, cf. panels E and F) by in situ hybridization to tissue sections, using a *PaxB* antisense RNA probe.

PaxB Binding to Pax2/5/8 and Pax6 Target Sites Is Affected in PaxB Specificity Mutants

We next studied binding of PaxB proteins to Pax2/5/8 and Pax6 DNA target sites by electrophoretic mobility shift assays (EMSA; Figure 2A). PaxB mutations, introduced at conserved residues of the paired domain, corresponded to missense mutations found in *PAX3* and *PAX6* of human patients with Waardenburg syndrome (WS), Aniridia (AN), and Peters' anomaly (Figure 2B). Two other PaxB mutations were a deletion of the octapeptide PaxB(OCTAΔ) and a triple mutation PaxB(IQN). The latter converts the Pax2/5/8-specific amino acids Q, R, and H of the paired domain to I, Q, and N specific for the Pax6-type paired domain (highlighted in red in Supplemental Figure S1), altering the DNA binding specificity of the PaxB paired domain to that of the Pax6 paired domain (Czerny and Busslinger, 1995). We used binding sites derived from target genes of Pax6 (G1, Ey, ZPE) and Pax2/5/8 (TPO, H2B-2.2, H2A-2.2, CD19-1) as well as the canonical Pax consensus binding site, PAXcon, or its derivatives carrying mutations in the regions bound by the RED or the PAI subdomain of the bipartite paired domain (Czerny et al., 1993; Xu et al., 1995, 1999), FOP-RED or FOP-PAI. Both wild-type PaxB and PaxB(OCTAΔ) bound well to all Pax6 or Pax2/5/8 binding sites (Figure 2A). Pax6 has been shown previously to recognize the H2B-2.2 but not the H2A-2.2 target site (Czerny and Busslinger, 1995). In accordance with this finding, PaxB(IQN) did not bind to the H2A-2.2 target site and thus may be considered a "Pax6 specificity" mutant. Unexpectedly, PaxB proteins carrying some of the mutations (R23G, S40P, G9R, N14S, N14H) retained partial or almost complete DNA binding activity for paired domain binding sites, while two of the mutations (P17L, I84R) did not bind any of the tested target sites (Figure 2A).

Wild-type PaxB stimulated the activity of the consensus Pax promoter about 10-fold (Figure 2C), while the abilities of the mutant PaxB proteins to activate this promoter closely correlated with their binding activities in EMSAs (bands at the top in Figure 2A). Accordingly, we have used PaxB(I84R) as a loss-of-function mutant in the following studies.

PaxB Has a Transactivation and an Inhibitory Domain Typical of the Pax2/5/8 Subfamily

In order to study the potential activation and/or repression functions of PaxB, we have fused different parts of its C-terminal region to the N-terminal DNA binding domain of the yeast Gal4 protein (Figures 3A and 3B). By cotransfecting the epithelial kidney cell line 293 with the various fusion constructs and a Gal4-responsive *luciferase* reporter gene (Figure 3C), we identified two transactivation domains in the C-terminal region of PaxB, G3 and G6, flanking an inhibitory domain (Figure 3D, left panel). The inhibitory domain suppresses the transcriptional activity of PaxB, as does the inhibitory domain of Pax5 (Figure 3D, right panel) used in Gal4(1–147)-Pax5 fusion constructs as a control (Figure 3A). When the Gal4 DNA binding domain was fused to the entire C-terminal region of Pax6 (Figure 3A) and used as an additional control in a transfection assay, strong transcriptional activation was observed (Figure 3D, right panel). Similar results were obtained using COS7 cells (data not shown). The observed transcriptional effects were due to intrinsic differences rather than differential stabilities of the fusion proteins because comparable amounts of the

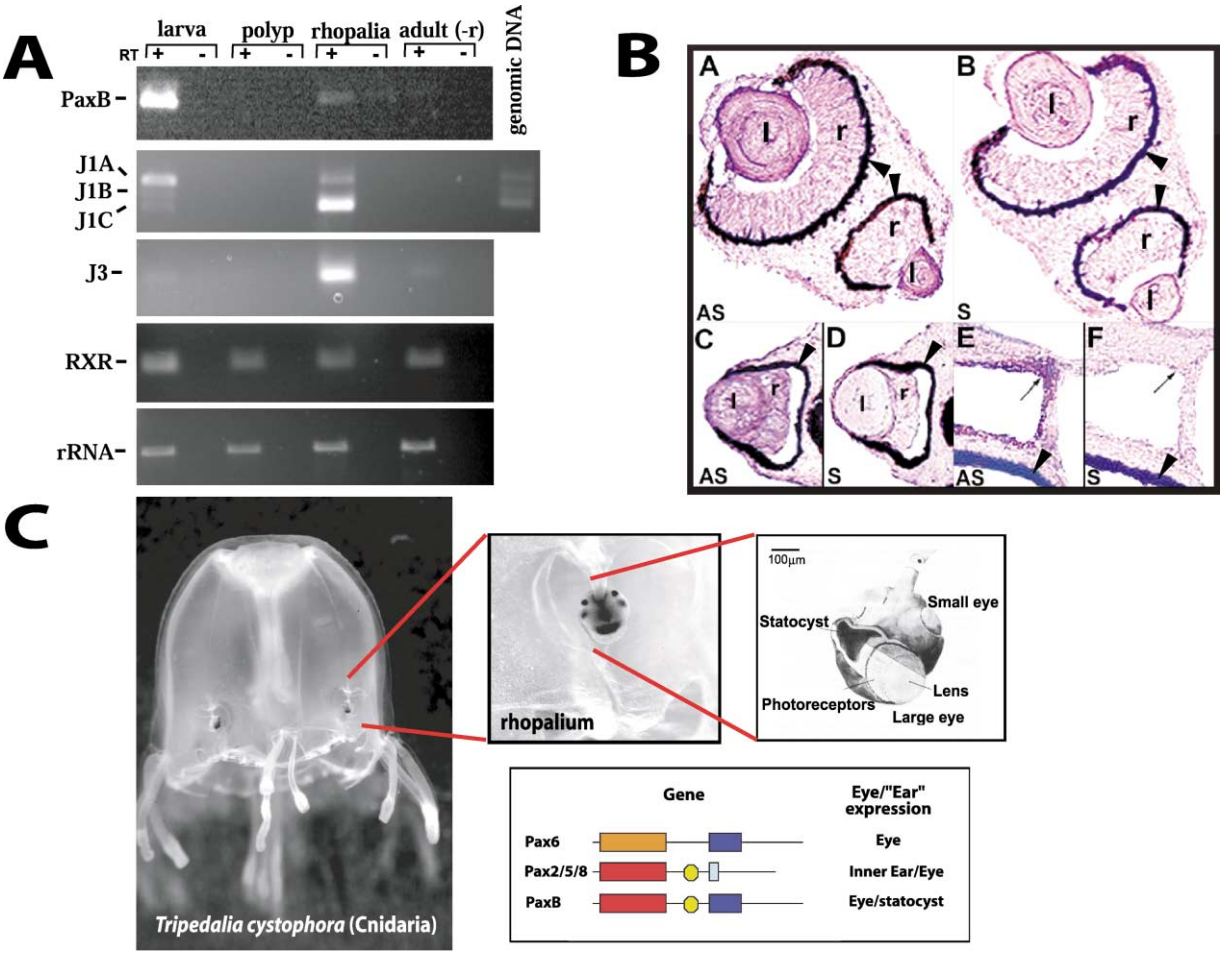

Figure 1. *PaxB* Gene Expression

(A) RT-PCR of *PaxB*, *J1A*-, *J1B*-, *J1C*-, and *J3*-crystallins, *RXR*, and *rRNA* RNAs.

(B) In situ hybridization of cryosections using *PaxB* antisense (AS; panels A, C, and E) and sense (S; panels B, D, and F) RNA probes. Arrows indicate staining in lumen of statocyst; arrowheads indicate pigmented layer.

(C) Medusa with rhopalium (left inset). Schematic drawing (right inset) of rhopalium. Bottom panel: paired domain (brown/red), octapeptide (yellow), and homeodomain (blue).

fusion proteins were detected by Western analysis of lysates of the transfected cells (Figure 3E). Thus, PaxB has a transactivation domain flanked by an inhibitory region, a typical feature of the Pax2/5/8 subfamily.

PaxB Is Unable to Induce Phosphorylation of Grg Transcriptional Corepressors

Pax2/5/8 proteins are converted from transcriptional activators to repressors by interaction with evolutionarily conserved transcriptional corepressors of the Groucho family (Eberhard et al., 2000). The hallmark of this interaction is the ability of these Pax proteins to induce phosphorylation of both mammalian Grg proteins and *Drosophila* Groucho. To test whether PaxB behaves like mammalian Pax2, we coexpressed mouse Grg4 protein and PaxB, mouse Pax2, or mouse Pax6 in COP-8 cells. While Pax2 induced a more slowly migrating band of Grg4, an indication of phosphorylated Grg4 (Figure 3F, asterisk), neither PaxB nor Pax6 induced a similar shift in the mobility of Grg4 protein. Therefore, PaxB appears to lack a key regulatory function of Pax2/5/8 family members despite the presence of a Pax2-type octapeptide, which is essential for the interaction of Pax2/5/8 proteins with Groucho (Eberhard et al., 2000).

PaxB Is an Efficient Transactivator of the *Drosophila rhodopsin* Gene

Drosophila Pax6 (Ey) directly activates expression of *rhodopsin* genes through homeodomain binding sites in the proximal region of their promoters (Sheng et al., 1997; Papatsenko et al., 2001). Consequently, we tested whether PaxB can transactivate a *lacZ* reporter gene under control of a *Drosophila* rhodopsin promoter. PaxB was a more efficient inducer of the promoter-driven reporter gene than was mouse Pax6 (Figure 4A). In contrast, mouse Pax2, which lacks a DNA binding homeodomain, did not activate the *rhodopsin* promoter (Figure 4A).

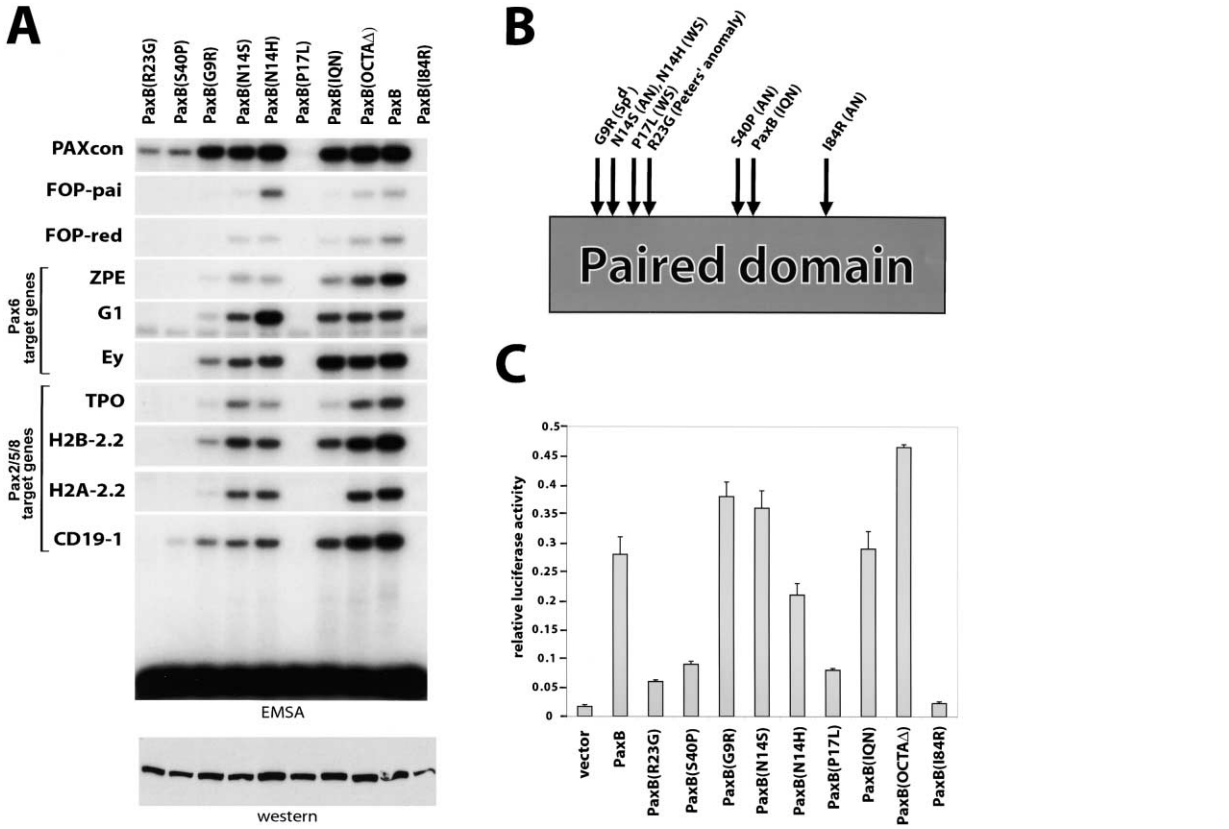

Figure 2. DNA Binding and Transactivation Properties of Wild-Type and Mutant PaxB Paired Domain

(A) Top panel: EMSAs. Bottom panel: Western blot with anti-FLAG M2 antibody.

(B) PaxB mutations. *Sp^d^*, Splotch delayed (*Pax3* mutation); *AN*, Aniridia (*PAX6* mutation); *WS*, Waardenburg syndrome (*PAX3* mutation); Peters' anomaly (*PAX6* mutation). PaxB(IQN) mutation converts the paired domain binding specificity of Pax2/5/8 to that of Pax6 (see Supplemental Figure S1).

(C) Transactivation of the *TPO-luc* reporter gene in cotransfected human epithelial kidney cell line 293.

PaxB Activates Jellyfish Crystallin Genes by Binding to Their Promoters

We tested whether PaxB can activate the *J1A-, J1B-*, and *J3-crystallin* promoters in cotransfected mammalian cells. Negative controls included the empty expression vector, the DNA binding-deficient PaxB(I84R) protein, and the promoter-less *luciferase* gene (*pGL3*); the *luciferase* gene driven by the *thyroperoxidase* (*TPO*) promoter, a well-established mammalian Pax8 target, served as a positive control (Figure 4B). Initial characterization by EMSA suggested the presence of PaxB binding sites in both *J1A-* and *J1B-crystallin* promoters (data not shown). PaxB stimulated luciferase activity driven by the *J1A-* or *J1B-crystallin* promoters 2- to 3-fold over that resulting from the promoter-less *luciferase* gene in the *pGL3* vector; this was 7- to 9-fold greater than the basal levels of luciferase activity in the absence of PaxB. More strikingly, PaxB induced the *J3-crystallin* promoter approximately 30-fold, about twice that of the *TPO* promoter (Figure 4B). PaxB(I84R) did not activate the *J3-crystallin* promoter, suggesting that promoter activation by PaxB depends on DNA binding through its paired domain.

Two Paired Domain Binding Sites in the Proximal Promoter of *J3-crystallin* Regulate Its Activation by PaxB

In order to identify PaxB response elements in the *J3-crystallin* promoter, we generated a series of *J3* promoter deletions in the *luciferase* reporter construct. The shortest promoter fragment, consisting of only 200 bp upstream of the transcriptional start site, was activated by PaxB and not by PaxB(I84R) (data not shown; but cf. Figure 5C). In the −66/−34 region of the TATA box-containing promoter, we identified two putative paired domain binding sites (Figures 5A and 5B) that fit well with the Pax2/5/8 paired domain consensus sequence (Czerny and Busslinger, 1995). Both potential Pax binding sites within the *J3-crystallin* promoter were mutated individually and in combination in the *luciferase* reporter gene (Figures 5A and 5B). Mutation of each site reduced reporter gene activation by cotransfected *PaxB* (Figure 5D), consistent with highly reduced PaxB binding affinities (Figure 5C, left and middle panels). Because binding site 2 (−50/−66) contains a C as the last nucleotide of the consensus sequence (asterisk in Figure 5B) and Pax6 strongly prefers A in that position (Czerny and

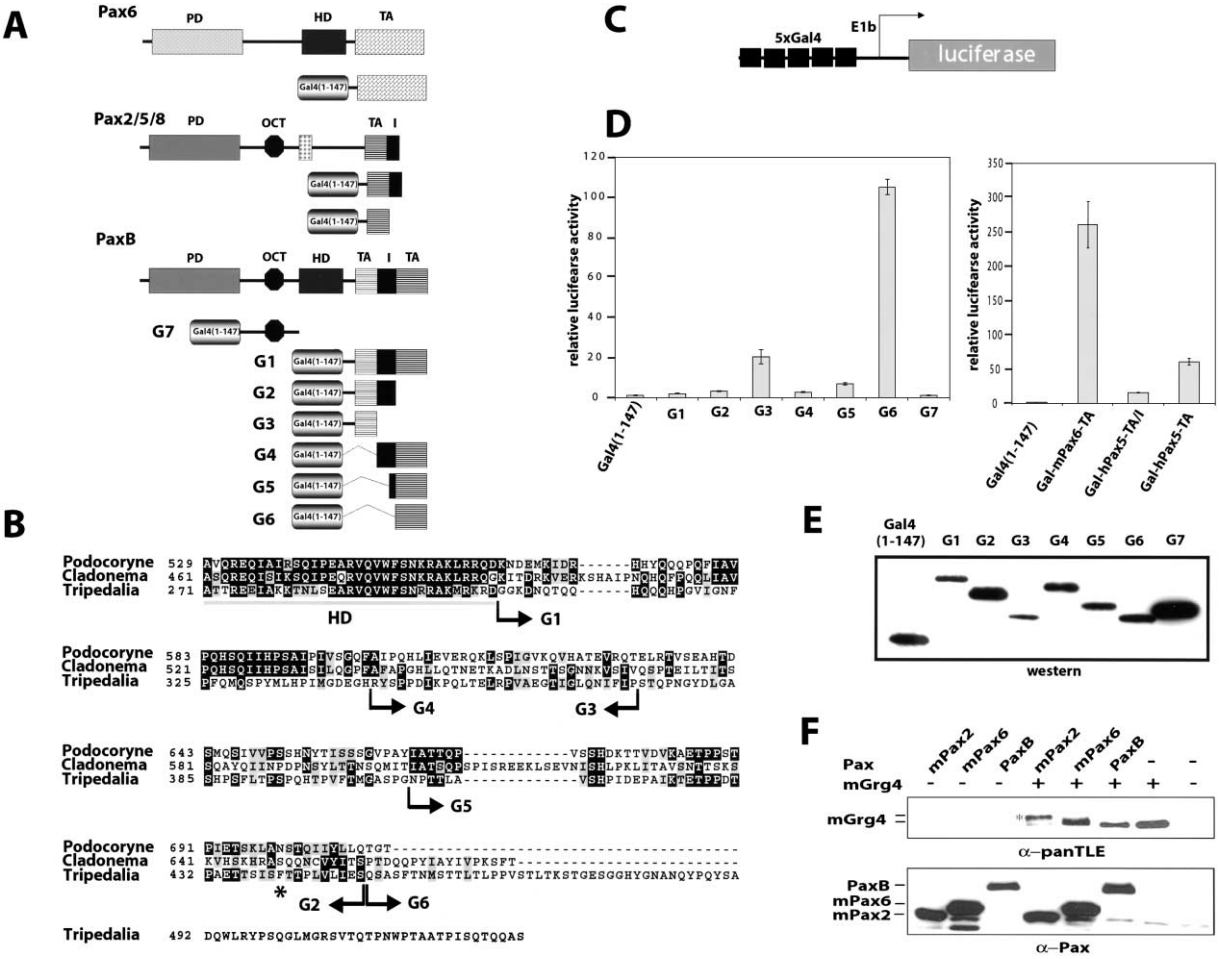

Figure 3. PaxB Has Adjacent Transactivation and Inhibitory Domains Typical of Pax2/5/8 Proteins, but Is Unable to Induce Grg Phosphorylation

(A–C) Schematic diagrams of Gal4 DNA binding-PaxB fusion constructs (A) and *luciferase* reporter gene (C) cotransfected into human epithelial kidney cell line 293. The positions of breakpoints for PaxB-specific fusions G1–G6 are shown in (B).

(D) PaxB contains two regions with intrinsic transactivation capacity TA, G3 and G6 (left histogram), separated by an inhibitory domain I (A). The inhibitory domain I adjacent to the transactivation domain TA is present in human Pax5; in contrast, Pax6 contains a strong activation domain TA (right histogram).

(E) Western blot of transfected cell lysates with anti-HA antibody.

(F) Western blot of whole cell extracts with anti-Grg (α-pan-TLE) and anti-paired domain (α-Pax) antibodies. The asterisk indicates Pax2-induced shift in Grg4 mobility.

Busslinger, 1995), we tested whether binding site 2 is recognized specifically by Pax proteins of the Pax2/5/8 subfamily and not by Pax6. Indeed, while both PaxB and mouse Pax2 bound to site 2, neither Pax6 nor the Pax6 specificity mutant of PaxB, PaxB(IQN), did (Figure 5C, right panel). We conclude that the promoter of the *J3-crystallin* gene is a direct target of PaxB and is activated through its binding of the PaxB paired domain.

Activation of the *J3-crystallin* Promoter Is Restricted to the Pax2/5/8 Subfamily

We next tested whether other members of the Pax protein subfamilies, especially Pax6, can activate the *J3-crystallin* promoter. Cotransfection with mouse *Pax2* increased *J3-crystallin* promoter activity, but 4-fold less than stimulation with *PaxB* (Figure 5E, left panel). By contrast, cotransfection with mouse *Pax1*, *Pax3*, or *Pax6* did not activate the *J3-crystallin* promoter (Figure 5E, left panel). Moreover, although PaxB(IQN) activated the promoter containing the general Pax binding site, it did not activate the *J3-crystallin* promoter in transfection assays (Figure 5E, right panels). Pax activation of the *J3-crystallin* gene promoter thus appears to be confined to the Pax2/5/8 subfamily.

Jellyfish PaxB Can Substitute for Pax2 Functions in the *Drosophila* Eye

To further examine the similarities between PaxB and Pax2, we tested whether PaxB can rescue the *Drosophila* eye mutant *spa*^{pol}, which results from the absence of *D-Pax2* expression in cone and primary pigment cells of developing larval and pupal eye discs (Fu and Noll, 1997). PaxB protein was expressed under the indirect control of the eye-specific enhancer *spa* of *D-Pax2* by

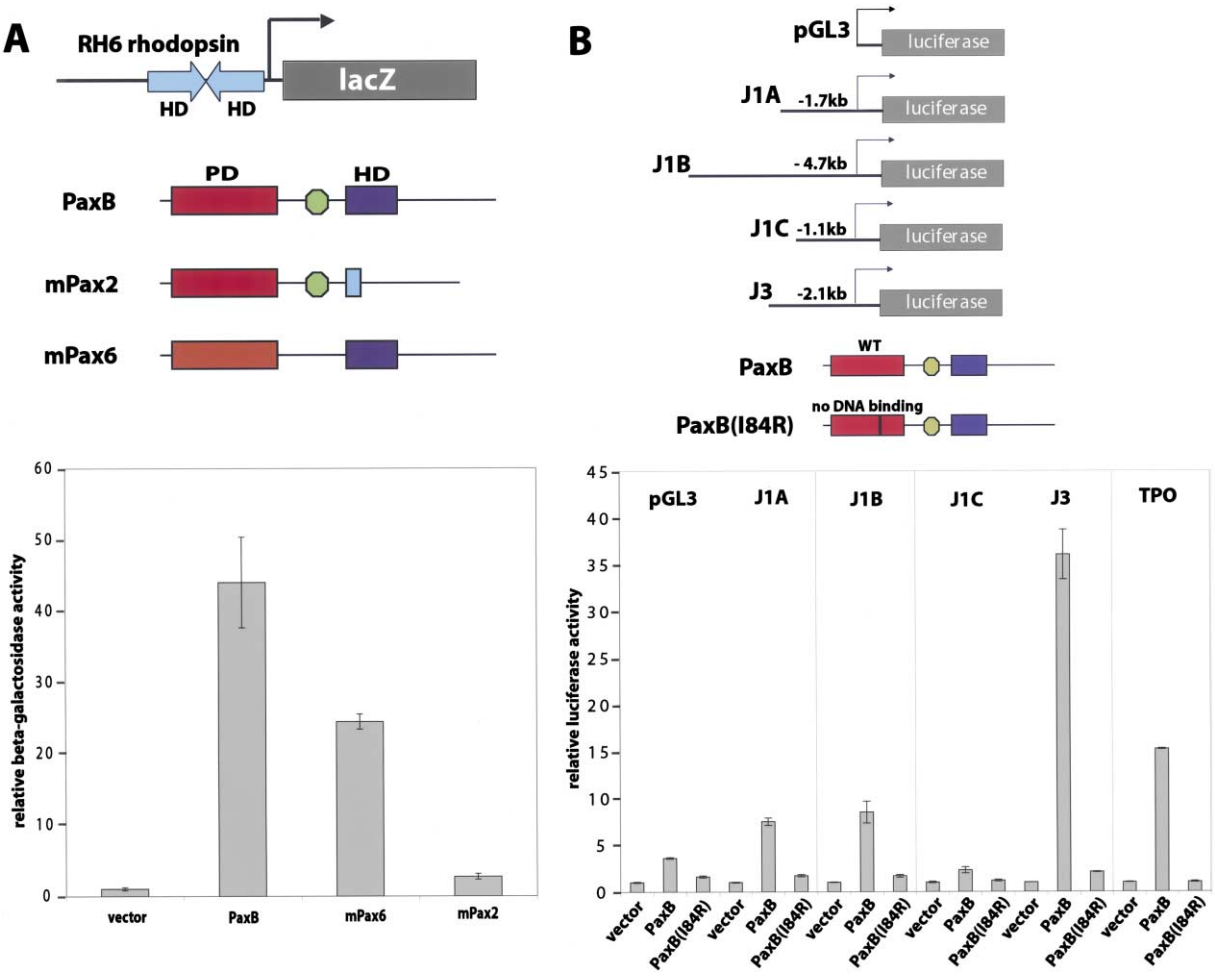

Figure 4. PaxB Transactivates *Drosophila rh6 rhodopsin* and *Tripedalia J1- and J3-crystallin* Promoters

(A) Expression vectors (top) cotransfected into COS7 cells with the *pRH6-lacZ* reporter gene (bottom).

(B) Expression vectors (top) cotransfected into 293 cells with the indicated jellyfish *crystallin-luciferase* reporter gene (bottom).

use of the *spa-Gal4* and *UAS-PaxB* transgenes. PaxB largely rescued the *spa*^{pol} phenotype (Figure 6C), which is indistinguishable from that shown in Figure 6D. The rescued eyes appeared similar to, but not quite as regular as, wild-type eyes (Figure 6A). However, the rescue was as efficient as that obtained with D-Pax2 expressed similarly under the indirect control of *spa-Gal4*, which also did not fully rescue the *spa*^{pol} eye phenotype (Figure 6B). In contrast, D-Pax2 expression under the direct control of the *spa* enhancer in a single transgene completely rescued the *spa*^{pol} phenotype (Flores et al., 2000).

PaxB(I84R), which does not bind DNA, did not rescue *spa*^{pol} (Figure 6D), and the eye phenotype was indistinguishable from that of *spa*^{pol} mutants (compare, for example, Figure 4D in Fu and Noll, 1997). To our surprise, PaxB(IQN), which has a Pax6 binding specificity, rescued the *spa*^{pol} phenotype (Figure 6E), but less efficiently than wild-type PaxB. In contrast, PaxB(OCTAΔ), which like Ey and Pax6 has no octapeptide, had a similar rescue efficiency (Figure 6F) as wild-type PaxB (Figure 6C).

We further investigated whether the two Pax6 proteins of *Drosophila*, Ey and Toy (Czerny et al., 1999), can also substitute for D-Pax2 functions in cone and primary pigment cells during eye development. Rescue of the *spa*^{pol} phenotype was generally similar for Ey (Figure 6G) and Toy (Figure 6H), although significantly less efficient than for PaxB (Figure 6C).

Jellyfish *PaxB* and *D-Pax2* Induce Ectopic Eyes in *Drosophila*

Because jellyfish PaxB can substitute for Pax2 in the *Drosophila* eye, we tested whether it can also substitute for Ey or Toy to induce ectopic eyes (Halder et al., 1995; Czerny et al., 1999). *UAS-PaxB* expressed ectopically under the control of *dpp-Gal4* induced one small ectopic eye in each tibia (Figures 7B and 7G), as did Ey or Toy when expressed under the same transcriptional control (Figures 7A and 7F). However, ectopic eyes were induced only in three out of the five *UAS-PaxB* lines that rescued the *spa*^{pol} phenotype (Figure 6C), and the eyes were smaller than those obtained by ectopic expression of Ey (Figure 7A) or Toy (Figure 7F). This suggests that ectopic eye induction requires a relatively high threshold of PaxB protein and that its activity is below that of Ey or Toy. As expected, when PaxB was replaced by

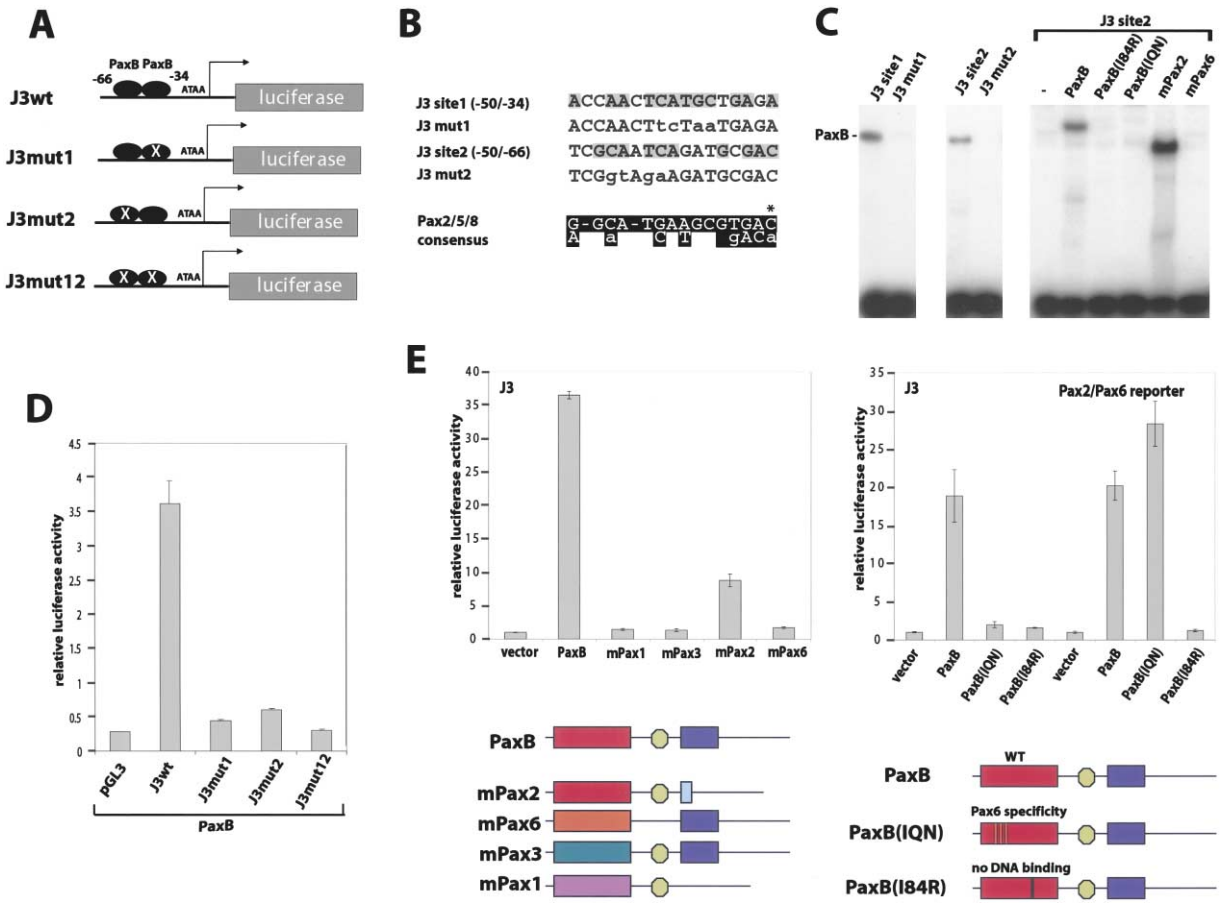

Figure 5. Paired Domain Binding Sites in the *J3-crystallin* Promoter Mediate Activation by PaxB

(A) *Luciferase* reporter genes with *J3-crystallin* promoter including two paired domain binding sites (X, mutated) and their activation by *PaxB* expression constructs cotransfected into 293 cells (D).

(B) Putative PaxB binding sites in *J3-crystallin* promoter; match with the Pax2/5/8 consensus sequence in gray; mutations are set lower case. The asterisk marks the position within the Pax consensus sequence responsible for differential binding affinity of Pax6 and Pax2/5/8.

(C) EMSA of different Pax proteins using *J3* sites 1 and 2 as probes.

(E) *J3-crystallin* promoter-*luc* (left panel) or 476Glu-*luc* (a Pax2/Pax6 promoter construct; right panel) reporter genes cotransfected with the indicated Pax expression vector into COS7 (left panel) and 293 (right panel) cells.

PaxB(IQN), the ectopic eyes were as big as those induced by Ey or Toy, and all six lines produced one small eye on each tibia (Figures 7C and 7H). However, in contrast to Ey but like Toy, PaxB(IQN) did not induce ectopic eyes in wings (not shown) or antennae (Figure 7A). Thus, PaxB(IQN) activity appears closer to Toy than Ey activity.

PaxB(OCTAΔ) induced ectopic eyes (Figures 7D and 7I) of similar size but with slightly lower efficiency than did wild-type PaxB. Only four of the five *UAS-PaxB(OCTAΔ)* lines that rescued the *spa*^{pol} phenotype produced a maximum of four ectopic eyes per fly. We conclude that PaxB can substitute for Pax2 and Pax6 functions in *Drosophila*.

Because Ey and Toy can substitute for D-Pax2 to rescue the *spa*^{pol} phenotype partially, we asked whether the reverse is also true, that is, can D-Pax2 replace some of the functions of Ey or Toy as assayed by the induction of ectopic eyes. Surprisingly, this was indeed the case. In one *UAS-D-Pax2* line, ectopic eyes were induced under the control of *dpp-Gal4* in the tibia (Figures 7E and 7J) as efficiently as in the three *UAS-PaxB* lines (Figures 7B and 7G). In contrast, other *Drosophila* paired domain proteins (Paired [Prd], Gooseberry [Gsb], Pox meso [Poxm], Pox neuro [Poxn]) did not induce ectopic eyes when similarly expressed under *dpp-Gal4* control. This finding suggests that of all *Drosophila* Pax proteins, only D-Pax2 has retained some of the Ey and Toy functions. It further follows that the Pax2/5/8 subfamily is most closely related to the Pax6 subfamily, whose ancestor we suggest was a PaxB-type paired domain protein.

Discussion

PaxB Unites Structural and Functional Features of Pax2 and Pax6

The present study shows that the structure of the *Tripedalia PaxB* gene, like that of other cnidarians (Sun et al., 1997, 2001; Gröger et al., 2000; Miller et al., 2000) and of a sponge (Hoshiyama et al., 1998), corresponds to an ancestral Pax gene, encoding a paired domain,

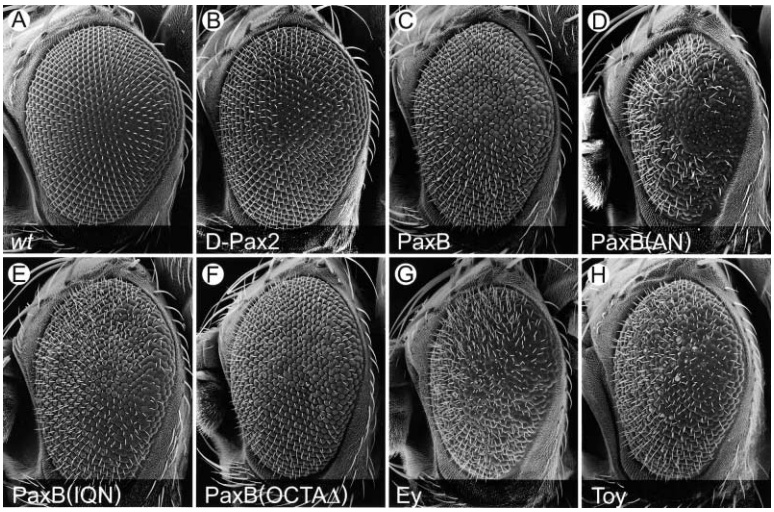

Figure 6. Rescue of the *spa*^{pol} Eye Phenotype by Jellyfish PaxB Expression under Control of the *spa* Enhancer

Left eyes of wild-type flies (A) and of offspring from crosses of *w spa-Gal4*; *spa*^{pol} virgins with males carrying homozygous *UAS-D-Pax2-1* (B), *UAS-PaxB* (C), *UAS-PaxB(AN)* (D), *UAS-PaxB(IQN)* (E), *UAS-PaxB(OCTAΔ)* (F), *UAS-Ey* (G), or *UAS-Toy* (H) transgenes (all in a *spa*^{pol} and *y w* or *w* background) are compared by scanning electron microscopy.

an octapeptide, and a homeodomain, as previously predicted (Noll, 1993; Balczarek et al., 1997). We show here that the PaxB protein is a functional hybrid of Pax2/5/8 and Pax6. On the basis of DNA sequence and DNA binding assays, it has been previously proposed that cnidarian *PaxB* has maintained the structure of a Pax gene ancestral to modern Pax6 and Pax2/5/8 (Sun et al., 1997, 2001). The sequence and DNA binding specificity of the PaxB paired domain of *Tripedalia* are characteristic for the Pax2/5/8 subfamily, the DNA binding specificity of which is generally broader than that of Pax6 proteins (this study; Sun et al., 2001). In addition, PaxB includes in its C terminus adjacent activation and inhibitory domains, a characteristic of Pax2/5/8 (Dörfler and Busslinger, 1996; Lechner and Dressler, 1996; Kreslová et al., 2002). By contrast, Pax6 contains a transactivation domain composed of short regions that act in synergy with each other (Tang et al., 1998). Importantly, PaxB can rescue the *Drosophila spa*^{pol} mutant whose eye-specific enhancer of *Pax2* is deleted (Fu and Noll, 1997). However, other properties of *Tripedalia* PaxB are clearly Pax6-like. First, unlike Pax2, PaxB does not induce phosphorylation of Grg4, a Groucho-type transcriptional corepressor that interacts with vertebrate Pax5 via the octapeptide (Eberhard et al., 2000). It seems unlikely that this negative result is caused by interspecies differences because various vertebrate Pax2/5/8 proteins and *Drosophila* D-Pax2 induce phosphorylation of *Drosophila* Groucho and mouse Grg4, which argues for an evolutionarily conserved mechanism (Eberhard et al., 2000). Second, like Pax6, PaxB has a *prd*-type homeodomain with a cognate DNA binding specificity. This was deduced from an even greater activation of the *Drosophila rh6* promoter by PaxB than authentic Pax6 (Sheng et al., 1997; Papatsenko et al., 2001) in transient transfection assays. Finally, even though PaxB

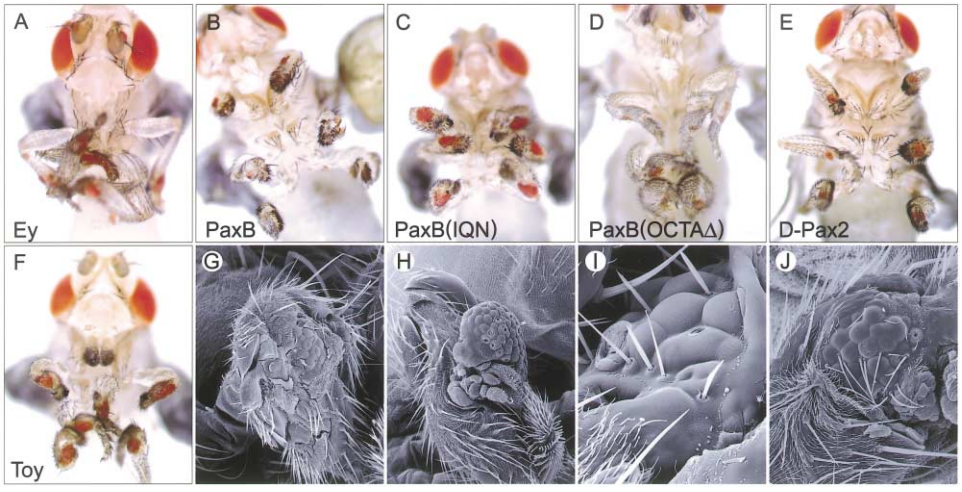

Figure 7. Induction of Eyes on *Drosophila* Legs by Ectopic Expression of PaxB or D-Pax2

Ventral views of heterozygous *dpp-Gal4* flies with ectopic eyes induced by a *UAS-Ey* (A), *UAS-PaxB* (B), *UAS-PaxB(IQN)* (C), *UAS-PaxB(OCTAΔ)* (D), *UAS-D-Pax2* (E), or *UAS-Toy* (F) transgene located on the other third chromosome are compared by light microscopy. Enlarged views of ectopic eyes from flies with the same genotype as shown in the panel above are compared by scanning electron microscopy (G–J). Relative magnifications: 130× (G and H), 1100× (I), and 400× (J).

rescued the *D-Pax2 spa^{pol}* mutant, it was also able to induce ectopic eyes in *Drosophila*, although with lower efficiency than PaxB(IQN). This is a striking difference from zebrafish Pax2, which is unable to generate ectopic eyes in *Drosophila* (Nornes et al., 1998).

Induction of ectopic eyes in *Drosophila* by PaxB appears intriguing, particularly if one considers that rescue of the *Drosophila ey^{2}* mutant requires a protein with a Pax6-type paired domain, quite different from the Pax2-type paired domain of jellyfish PaxB, but not a homeodomain (Punzo et al., 2001). It is less surprising, however, in view of our finding that D-Pax2 is also able to induce ectopic eyes. Moreover, induction of ectopic eyes depends on a wild-type endogenous *ey* gene, which is initially activated by Toy (Czerny et al., 1999) and subsequently maintains the genetic program for eye development by positive feedback loops (Chen et al., 1997; Pignoni et al., 1997). Accordingly, PaxB needs to bind to the Pax6 binding sites of the *ey* enhancer to induce ectopic eye development. This requirement is improved by altering the binding specificity of PaxB to that of Pax6 in PaxB(IQN), which is as efficient as Toy in its capacity to induce ectopic eyes. Our results, therefore, suggest that Pax6 target sites of the *ey* enhancer are recognized by PaxB and even D-Pax2, when expressed at high levels, with affinities that suffice to turn on the program of eye development in a few susceptible cells of the leg disc that would normally form tibial structures. Although D-Pax2 can induce ectopic eyes, other *Drosophila* Pax proteins (Gsb, Prd, Poxm, and Poxn) are unable to do so, which implies that D-Pax2 is more closely related to PaxB than Poxn or any of the Pax proteins of the Pax3/7 and Pax1/9 subfamilies. Conversely, Toy or Ey are both able to rescue the *spa^{pol}* phenotype to some extent, which indicates that these Pax6 proteins are still able to perform some of the Pax2 functions. It follows that the Pax2 and Pax6 proteins have retained the capability to substitute for some of each other's functions. One implication of this is that eyes (ocelli) and future ears (statocysts/mechanoreceptors), which both express *PaxB* in *Tripedalia*, are developmentally and evolutionarily linked.

A PaxB-like Gene Was an Early Regulator of Crystallin Genes

Our data support the idea that a PaxB-like gene was a primordial regulator of lens crystallin genes. Crystallins differ from opsins by being taxon-specific, nonhomologous, distinct proteins with analogous functions, generally derived from or identical to enzymes or stress proteins (Wistow and Piatigorsky, 1988; de Jong et al., 1989; Tomarev and Piatigorsky, 1996).

Similar transcription factors (i.e., Pax6, Maf, and Sox family members, among others) have been implicated in the regulation of the nonhomologous crystallin genes (see Cvekl and Piatigorsky, 1996; Duncan et al., 2004). Pax6 activates or represses many vertebrate crystallin promoters in transient transfection assays. Coregulators may strongly enhance activation by Pax6, as does SOX2 for the chicken δ*1-crystallin* enhancer (Kamachi et al., 2001). While less is known about invertebrates, the promoter of the scallop *aldehyde dehydrogenase/*Ω*-crystallin* gene has an arrangement of putative *cis*-control elements, including Pax6 target sites, surprisingly similar to that used for lens promoter activity of the unrelated mouse and chicken α*A-crystallin* genes (Carosa et al., 2002).

Here we show that the *J3*- and possibly *J1-crystallin* genes of *Tripedalia* are regulated by PaxB via two adjacent paired domain binding sites just upstream of the TATA box. This arrangement is reminiscent of Pax2/5/8 binding sites in the promoters of the tissue-specific *H2A-2* and *H2B-2* histone genes in sea urchins (Barberis et al., 1989). Pax5 and Pax8 binding sites are also present close to the transcriptional start sites in the promoters of mouse and human B cell-specific *CD19* (Kozmik et al., 1992) and rat *TPO* (Zannini et al., 1992) genes. Site 2 of the *J3-crystallin* promoter shows a clear preference for Pax2/5/8 proteins and is not recognized by Pax6. Accordingly, of all mammalian Pax proteins tested (mouse Pax1, Pax2, Pax3, and Pax6), only Pax2 activated the jellyfish *J3-crystallin* promoter. Thus, *J3-crystallin* gene regulation in this jellyfish has been optimized for *PaxB*, apparently the only Pax gene present in this ancient species containing eyes. Taken together, our results suggest that *PaxB*, like *Pax6* in Bilateria, played a major role in the evolutionary recruitment of distinct multifunctional proteins to serve as lens crystallins.

Pax6 Is Not a Universal Regulator of Eye Development

The apparent absence of a *Pax6* gene in the eye-containing jellyfish *Tripedalia* (this study) and *Cladonema californicum* (Sun et al., 2001) questions the universality of a *Pax6* requirement for eye development (Gehring and Ikeo, 1999; Gehring, 2002). Cnidaria are the most basal phylum of the animal kingdom that has evolved eyes. Indeed, the eye structures in Cnidaria vary from simple eyespots to complex ocelli containing a cornea, lens, and retina (see Piatigorsky et al., 1989). These ocelli are similar to those found in other invertebrates and resemble the complex eyes of vertebrates (Land and Nilsson, 2002). *PaxB* expression in the jellyfish lens and retina, coupled with its ability to activate the *Drosophila* rhodopsin *rh6* and jellyfish crystallin (especially *J3-crystallin*) promoters, strongly suggests that *PaxB* performs functions required for eye development similar to those exercised by *Pax6* in higher metazoans.

We speculate that one of the first functions of the ancestral *PaxB* in eye evolution was the regulation of *rhodopsin* genes in primitive photoreceptors, which after duplication of the ancestral *PaxB* in the triploblast branch was taken over by the emerging *Pax6* that expanded its functions to include the morphogenesis of divergent eye structures later in evolution. It was proposed that intercalation of genes in the pathway between the master control gene *Pax6* and the bottom-of-the-cascade genes (*rhodopsins*) during evolution might explain the different morphological appearances of the various extant eye types (Gehring and Ikeo, 1999). Indeed, a series of elegant experiments has shown that the *Drosophila* homologs of *Pax6*, *ey*, and/or *toy* directly regulate expression of the *rhodopsin* genes *rh1*, *rh3*, *rh5*, and *rh6* in the photoreceptor cells through the palindromic RCSI/P3 site that is recognized by the homeodomain of Pax6 (Sheng et al., 1997; Papatsenko et al.,

2001). We have detected *PaxB* expression throughout the retina of both small and large adult ocelli of *T. cystophora* and have shown that PaxB can activate the *Drosophila rh6* promoter, rendering *PaxB* a plausible candidate for *rhodopsin* gene regulation in the jellyfish. It remains to be seen whether the authentic *Tripedalia rhodopsin* promoter is under PaxB control. However, apart from *Drosophila*, there is scant evidence that *Pax6* regulates *rhodopsin* gene expression in all species. It is not expressed in mouse photoreceptor cells (Davis and Reed, 1996), in developing adult eyes of a polychaete annelid (Arendt et al., 2002), or in regenerating planarian eyes (Pineda et al., 2002). The conclusion that Pax6 may not be a universal transcriptional regulator of *rhodopsin* genes is consistent with the hypothesis that PaxB, not Pax6, was the first *rhodopsin* gene regulator.

Evidence for Independent Evolution of Eyes in Cnidaria and Bilateria

We propose that the ancestral gene *PaxB* was responsible for eye development in cnidarians, suggesting that it was the primordial gene for eye evolution, and that *Pax6* arose from a common ancestor with *PaxB* in higher metazoans only after the separation of Cnidaria from Bilateria. This ancestral gene encoded a Pax2-like paired domain and octapeptide, and a *prd*-type homeodomain similar to modern *PaxB* in *Tripedalia*. In higher metazoa, the ancestral gene duplicated to generate *Pax2* and *Pax6*. Pax2 lost most of its homeodomain but retained the octapeptide, while Pax6 conserved the *prd*-type homeodomain but lost the octapeptide.

This hypothesis is suggested strongly by an analysis of paired domains of the Pax2/PaxB and the Pax6 subfamilies (Supplemental Figure S2). At 17 positions, cnidarian PaxB and arthropod/chordate Pax2/5/8 paired domains are identical or conserved but deviate from Pax6 paired domains, whereas no position exists at which cnidarian PaxB and arthropod/chordate Pax6 paired domains are conserved but deviate from Pax2/5/8 paired domains (Supplemental Figure S2). It follows that the ancestral paired domain of Pax2 and Pax6 required selection for many characteristic changes to evolve into a Pax6-type paired domain, while it required selection for only few changes to evolve into modern Pax2 or PaxB. The main difference among modern Pax proteins responsible for eye development thus appears to be the acquisition of a different and narrower DNA binding specificity of Pax6 by the mutations Q42I, R44Q, and H47N, and the loss of the octapeptide. That these mutations were essential is evident from our experiments demonstrating that PaxB(IQN) is as efficient as Toy and much more efficient than PaxB or *Drosophila* Pax2 in generating ectopic eyes in *Drosophila*.

While it has been proposed that a *PaxC* gene isolated from corals is the homolog of *Pax6* (Miller et al., 2000), this is not supported by phylogenetic tree analysis (Gröger et al., 2000; Miller et al., 2000; Sun et al., 2001), which suggests that PaxC is more closely related to cnidarian PaxB. Our analysis agrees with the notion that PaxC is more closely related to PaxB than to Pax6 (Supplemental Figure S2): the paired domain of PaxC deviates from that of Pax6, but is identical to that of Pax2 at ten positions where the Pax6 and Pax2 subfamilies have been conserved; the opposite situation is found only at four positions. It follows that PaxC is not a Pax6-like gene in terms of its paired domain structure but rather a Pax protein that diverged from PaxB after its duplication.

These considerations strongly support our hypothesis that Pax6 evolved by duplication from an ancestral PaxB-like gene only after the separation of Bilateria from Cnidaria. This raises the possibility that complex eyes arose independently in some cnidarians and higher metazoans, possibly within a relatively short time at the beginning of the Cambrian period (Nilsson and Pelger, 1994). Such an interpretation is consistent with that of Land and Nilsson (2002), who recently favored the idea that eyes were invented several times and today show different levels of homology among them.

Experimental Procedures

Collection of Jellyfish

T. cystophora were collected in the mangroves of La Parguera, Puerto Rico. The rhopalia were excised and stored at −80°C, as described (Piatigorsky et al., 1989).

Genomic Libraries and cDNA Clones

A paired box fragment, amplified from genomic DNA with degenerated primers corresponding to amino acid sequences YYETG and WEIRD (Gröger et al., 2000), was used to isolate and sequence a 3.7 kb EcoRI fragment from a genomic library (Piatigorsky et al., 2001). A 725 bp RT-PCR *PaxB* cDNA was amplified from rhopalia mRNA, using primers corresponding to genomic sequences of the paired domain (5′-gttgggaggagtattcgtcaatgg-3′) and homeodomain (5′-agatccgtttgctccggcgtaaag-3′), extended by SMART RACE (Clontech) using primers 349A (3′ RACE) and 349B (5′ RACE) (see Supplemental Data for all oligonucleotide primers), sequenced (GenBank accession number AY280703), and cloned into the pCR2.1 vector (Invitrogen). Genomic clones of *J1A*-, *J1B*-, and *J1C-crystallins* were isolated (Piatigorsky et al., 1993). A PCR amplification strategy was used to obtain genomic clones containing the *J1C*- and *J3-crystallin* promoters (Universal Genome Walker, Clontech). The *J1C-crystallin* fragment, amplified with primers 379A and the AP1-adaptor primer (Clontech kit), and the *J3-crystallin* fragment, amplified with primers 431B and AP1, were cloned into the pCRII-TOPO vector (Invitrogen).

RNA Isolation and RT-PCR Analysis

Total RNA was prepared using Trizol reagent (GIBCO-BRL). Single-stranded cDNA was obtained from 1 μg of total RNA using random hexamer primers and PowerScript (Clontech). cDNA synthesis was normalized with respect to rRNA by using cDNA amounts that produced equal amplification of rRNA transcripts in subsequent PCR-based expression analysis.

The following primer pairs were used to make *T. cystophora* cDNAs: 462A/D (rRNA), 349D/351A (*PaxB*), 465A/464 (*J1A*), 465B/464 (*J1B*), 465C/464 (*J1C*), 378B/463 (*J3*), and 466A/B (*RXR*; Kostrouch et al., 1998).

In Situ Hybridization

In situ hybridizations were performed as described (Wilkinson and Nieto, 1993). Jellyfish were fixed overnight in a 1:1 mixture of sea water and fixative (4% paraformaldehyde, 0.6 M NaCl, 150 mM phosphate buffer [pH 7.4]), transferred into ascending concentrations of methanol, and stored in 100% methanol at 20°C. Fixed jellyfish were transferred through descending concentrations of methanol into diethyl pyrocarbonate-treated PBS, then into 20% sucrose in PBS, and finally into OCT compound (Fisher Scientific). Overlapping *PaxB* cDNAs, generated by PCR using primer pairs 349A/504B and 505A/500B, were cloned into pBluescript to generate digoxigenin-labeled probes.

DNA Constructs

The reporter genes *TPO-luc*, *476Glu-luc*, and *Gal5E1*β*-luc* and expression vectors encoding *mPax1*, *mPax2*, *mPax3*, *mPax6*, *mGrg4*, *Gal(1–147)*, *Gal-mPax6TA*, *Gal-hPax5TA/I*, and *Gal-hPax5TA* were described previously (Czerny and Busslinger, 1995; Dörfler and Busslinger, 1996; Hill et al., 1999; Eberhard et al., 2000; Kreslová et al., 2002). The following primer pairs were used to make the promoters: 331A/C (*J1A*), 332A/C (*JIB*), 461A/B (*J1C*), and 487A/D (*J3*). Reporter genes were inserted into the pGL3 Basic vector (Promega). *J3* promoter deletions were generated by primers 560A, 560B, and 560C (5′ primers) used in conjunction with primer 487A (3′ primer). The *J3* reporter gene mutants mut1, mut2, and mut12 were produced by using the Quick-Change kit (Stratagene) and the primers 571, 576C, and 571 plus 576C, respectively. The *rhodopsin* reporter gene *pRH6-lacZ* was generated by subcloning the *rh6* promoter from pBS-RH6 (provided by D. Papatsenko) into pCASPER. The *PaxB* mammalian expression construct was generated by cloning a cDNA fragment (amplified with primers 349A and 506A) into the pKW vector containing the N-terminal FLAG-epitope. Site-directed mutagenesis of *PaxB* cDNA was performed by the Quick-Change kit (Stratagene). For the *Gal4-PaxB* fusion constructs G1 to G7, primers 505A/506A (G1), 505A/506B (G2), 505A/506C (G3), 505B/506A (G4), 505C/506A (G5), 505D/506A (G6), and 537A/B (G7) were used and the resulting cDNA fragments were inserted into a pKWGal4 vector containing an HA-epitope tag.

Cell Transfection, Luciferase Reporter Assays, Western Blotting, and EMSA

Cell culture procedures and luciferase and electrophoretic mobility shift assays (EMSAs) were described previously (Carosa et al., 2002). Nuclear extracts were prepared according to Schreiber et al. (1989). The final concentration of the binding reaction was 4% Ficol, 10 mM Tris-HCl (pH 8), 1 mM DTT, 1 mM EDTA, 100 mM KCl, 50 μg/ml poly(dI-dC). Oligonucleotides used for EMSAs were a Pax consensus binding site (PAXcon, oligonucleotide 320), RED domain mutation (FOP-RED, 347), PAI domain mutation (FOP-PAI, 348), rat *glucagon* site G1 (G1, 449), *Drosophila eyeless* (Ey, 397), guinea pig ζ*-crystallin* (ZPE, 401), rat *thyroperoxidase* (TPO, 396), *CD19* site 1 (CD19-1, 395), and sea urchin histone genes sites *H2B-2.2* (399) and *H2A-2.2* (400). Oligonucleotides used for detection of PaxB binding to *J3-crystallin* promoter sequences were 555A (*J3* site1), 555C (*J3* mut1), 576A (*J3* site2), and 576C (*J3* mut2).

Expression of Pax, Gal4, and Grg proteins in transfected cells was detected by Western blotting and the use of anti-FLAG M2 (Sigma), anti-paired domain (Adams et al., 1992), anti-HA (Roche Molecular Biochemicals), and anti-pan-TLE (Stifani et al., 1992) antibodies.

Cells were transiently transfected with the firefly-based reporter gene, the *Renilla* luciferase control plasmid *pRL-SV40* (Promega), and the indicated *PaxB* expression vector, using FuGENE6 (Roche Molecular Biochemicals). The total amount of transfected plasmid DNA was 1 μg/well of a six-well plate. After 2 days, luciferase activity was assayed with the dual-luciferase assay kit (Promega). Expression of the *pRH6-lacZ* reporter in transfected cells was measured by the β-galactosidase luminescent kit (Clontech) and a Tropix TR717 microplate luminometer (Applied Biosystems). *pCMV-luc* plasmid was used for normalization of transfection efficiency.

Generation of Transgenic Flies and Fly Stocks

Transgenic flies are described in Supplemental Data at http://www.developmentalcell.com/cgi/content/full/5/5/773/DC1.

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